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Effect of Neonatal Exposure to Diethylstilbestrol and Tamoxifen on Pelvis and Femur in Male Mice

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ABSTRACT ***Background:*** **Permanent abnormalities have been reported in reproductive and non-reproductive organs of mice and humans exposed perinatally to a synthetic estrogen, diethylstilbestrol (DES). Recent studies demonstrated that sex hormones affected the shape of the innominate bone in mice. Therefore, we analyzed the long-term effects of neonatal exposure of DES and tamoxifen, an anti-estrogen, in mouse bones.**

Methods: **Changes in the pelvis and femur were examined in 1- to 15-month-old C57BL/Tw male mice given 5 daily injections of 3 μg DES or of 100 μg tamoxifen beginning on the day of birth by measuring contents of calcium (Ca) and phosphorus (P), and the numbers of osteoblasts and osteoclasts.**

Results: **The ash weight of pelvis and femur in neonatally DES- and tamoxifen-treated mice was lower than that in the controls at 2–15 months of age. Contents of Ca and P of pelvis and femur in neonatally tamoxifen-treated mice were lower than in the controls and neonatally DES-treated mice. In neonatally DES-treated mice at 6–12 months, Ca and P contents in the pelvis were lower than in controls, but not different in the femur. The number of osteoblasts per unit length of endocortical surface of the femur in 2- and 3-month-old DES- and tamoxifen-treated mice was lower than that in the controls. The osteoclast number in the femur in DES-treated mice at 2 to 12 months was not different from that in the controls; however, in tamoxifen-treated mice, the number was higher than in the controls. An epiphyseal line was clearly detected in the femur of 12- and 15-month-old DES- and tamoxifen-treated male mice, whereas the line in the controls disappeared after 12 months.**

Conclusions: **The present results indicate that in male mice, neonatal exposure to DES and tamoxifen induced permanent changes in the pelvis and the femur, and that tamoxifen had a greater effect on bone tissue than did DES.**

Key words: Femur, Pelvis, Neonatal treatment, Tamoxifen, Diethylstilbestrol, Mouse

Short-term perinatal exposure of mice to estrogens including the synthetic estrogen, diethylstilbestrol (DES) induces irreversible changes in estrogen target tissues: persistent proliferation of vaginal epithelium and metaplasia of uterine epithelium (Takasugi et al., 1962; Takasugi, 1963; Takasugi and Bern, 1964; Iguchi et al., 1985; for reviews see, Takasugi, 1976, 1979; Bern and Talamantes, 1981; Mori and Iguchi, 1988; Iguchi, 1992), polyovular follicles (Forsberg et al., 1985; Iguchi, 1985; Iguchi et al., 1986), decrease in spermatogenesis (Takasugi, 1971; Takasugi et al., 1983), and epithelial changes in the seminal vesicle, epididymis, and prostate (McLachlan et al., 1975; Jones, 1980; for reviews see McLachlan, 1979; Arai et al., 1983). In humans, clear cell adenocarcinoma of the vagina has been reported in young women whose mothers were exposed to DES during pregnancy (Herbst et al., 1971; for reviews see Herbst and Bern, 1981; Mori and Nagasawa,

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1988). Perinatal injections of tamoxifen, an anti-estrogen, also induces permanent abnormalities in male and female mouse reproductive and non-reproductive organs (for review see Iguchi, 1992).

Recent studies showed that sexual dimorphism of the mouse pelvis is induced by sex steroids (Iguchi et al., 1989; Iguchi, 1992; Uesugi et al., 1992a,b); bone turnover is modified by estrogen in ovariectomized rats (Wronski et al., 1988). Total calcium (Ca) content of the tibia and lumbar vertebrae in castrated male rats is lower than in the normal males, but the content increases after estrogen administration (Vanderschueren et al., 1992). However, estrogen does not stimulate cancelous bone formation in female rats (Westerlind et al., 1993; Turner et al., 1994). Estrogen receptors have been demonstrated in human and rat osteoblast-like cells (Eriksen et al., 1988; Komm et al., 1988) and mouse periosteal cells (Iguchi, 1992; Uesugi et al., 1992b). Neonatal exposure of mice to DES did not change the shape of the pelvis, although tamoxifen induced abnormal development of the pelvis (Iguchi et al., 1988; Uesugi et al., 1993) and the os penis (Iguchi et al., 1990). Migliaccio et al. (1992) demonstrated that neonatal treatment of female mice with DES reduced the total amount of Ca and the weights of lumbar vertebrae and femurs, resulting in permanent changes in bone tissue in adulthood. These findings suggest that short-term exposure to estrogen and anti-estrogen during the perinatal period causes irreversible changes directly or indirectly in mouse bones. The present study, therefore, was planned to analyze the long-term effects of DES and tamoxifen on the pelvis and femur by examining the alteration of both cellular and mineral components in male mice exposed neonatally to DES and tamoxifen. Blood testosterone levels were also measured.

MATERIALS AND METHODS

Animals

Male C57BL/Tw mice kept under 12 h light/12 h dark at 23–25°C temperature were given standard laboratory chow (CE-2, CLEA, Tokyo) and tap water ad libitum. All mice were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals, as approved by our institutional animal care committee.

Animals were treated neonatally with 3 μg DES (Sigma Chemical Co., St. Louis, MO) dissolved in 0.02 ml sesame oil, 100 μg tamoxifen (Sigma) suspended in 0.02 ml saline or saline alone for 5 days from the day of birth. Animals were killed by decapitation at 1, 2, 3, 4, 6, 12, and 15 months of age. At autopsy, body weight was recorded and femurs and pelves were bisected: the right bones were used for histology and the left bones were used for measurement of minerals. Blood samples for the measurement of minerals in plasma were collected into heparinized sample tubes by decapitation under ether anesthesia and immediately centrifuged at 3,000 rpm for 15 min. For serum testosterone measurements, blood samples at 3 and 6 months of age were collected into nonheparinized sample tubes and immediately centrifuged at 3,000 rpm for 15 min. The plasma and serum were stored at −80°C until use. Eight mice were used for each time point.

Bone Histomorphometry

Right femur and pelvis (innominate bone) were fixed in 0.1 M Tris-HCl buffer containing 10% formalin and 10% ethylenediamine/tetraacetic acid (EDTA) for 2 days, decalcified in 0.1 M Tris-HCl buffer containing 10% EDTA, and then embedded in paraffin. Longitudinal (pelvis and epiphysis of femurs) or cross (center part of femur) sections cut with a microtome at 8 μm were stained with hematoxylin and eosin. The number of osteoblasts and osteoclasts per unit length (1 mm) of endocortical surface of femur and pelvis were determined by histomorphometrical methods (Uesugi et al., 1993) using an Image Analyzer (Olympus, Tokyo).

Minerals in Bones and Plasma

Left femur and pelvis (innominate bone) were cleaned from the surrounding tissues. Bones were dehydrated with absolute ethanol for 12 h and defatted with ether for 12 h. Bones were weighed before (wet weight) and after drying at 100°C for 2 days (dry weight); bone length was measured with calipers after drying. The dried bones were ashed at 600°C for 24 h in a muffle furnace (Shibata SMS-200; Tokyo); the ashed bones were weighed (ash weight) and dissolved in 0.5 ml 5 M HCl. The solution was diluted 180 times for Ca or 100 times for phosphorus (P) measurements in ion-exchanged water. Total amounts of Ca and P were measured using Calcium E-test Wako (Wako Pure Chemical, Osaka) by the method of Kazama et al. (1990) and P-test Wako (Wako) by the method of Taussky and Shorr (1953). Amounts of Ca and P in bones were normalized to the length (mg/mm length) and the ash weight of bones (mg/mg ash weight). Plasma Ca and P were also measured. Data expressed as the mean and standard error were analyzed by the Student *t*-test.

Testosterone Determination

Serum testosterone levels were determined by using an immunoassay kit (TESTOK-125-100: CIS Diagnostic, Saclay, France). The antiserum used reacted with testosterone, 5α-dihydrotestosterone, androstenedione, dehydroepiandrosterone, and other steroids to the extent of 100, 7.2, 0.81, 0.036, and less than 0.025%, respectively. Therefore, chromatographic separation was not carried out. The interassay variation was 9.0% and the intrassay variation was less than 10%. The least detectable T dose in this assay was 12.5 pg/tube. Individual testosterone value was the mean of duplicate determinations and was expressed as nanograms per milliliter of serum.

Statistical comparisons were performed either by the Student *t*-test or by Aspin Welch's *t*-test. Significant levels were $P < 0.05$.

RESULTS

Body Weight

The body weight of control male mice reached a plateau at about 4 months of age. Neonatally DES- and tamoxifen-treated mice were significantly lighter than the controls at all ages examined. Tamoxifen-treated mice were lighter in body weight than DES-treated mice (Fig. 1).

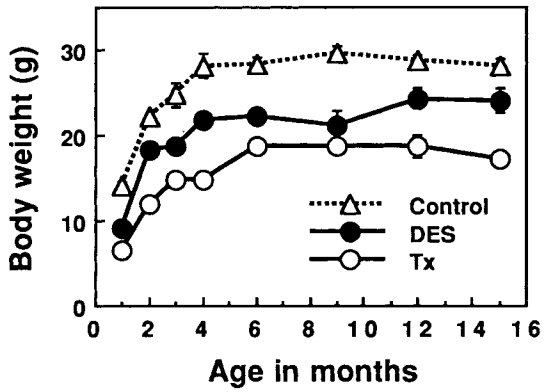

Fig. 1. Changes in body weight in control, neonatally DES- and tamoxifen (Tx)-treated male mice.

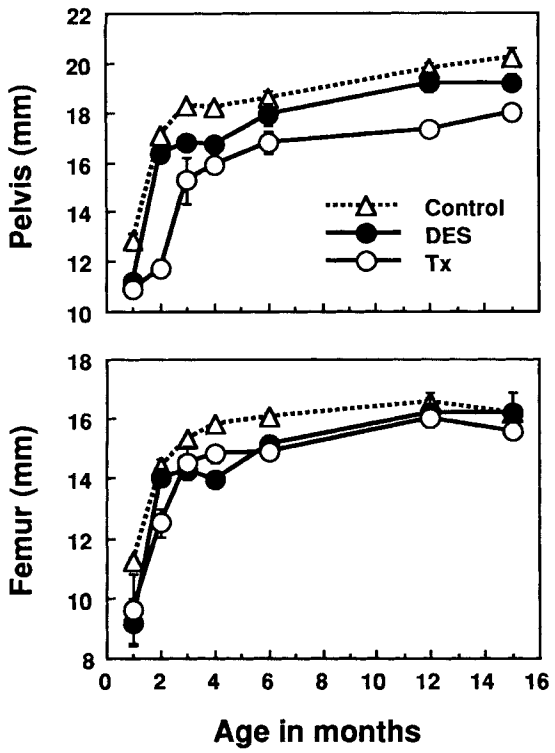

Fig. 2. Changes in length of pelvis (**upper**) and femur (**bottom**) in control, neonatally DES- and Tx-treated male mice.

Bone Length and Ash Weight ·

The lengths of pelvis and femur in control mice reached a plateau at 3 and 4 months of age, respectively (Fig. 2). Pelvis length in DES- and tamoxifen-treated mice was significantly shorter than that of the age-matched controls, whereas the length in tamoxifen-treated mice was significantly shorter than in DES-treated mice except at 1 month of age. At 2–6 months of age, the femur length of DES-treated and tamoxifen-treated mice was smaller than the age-matched controls. There was no difference in fe-

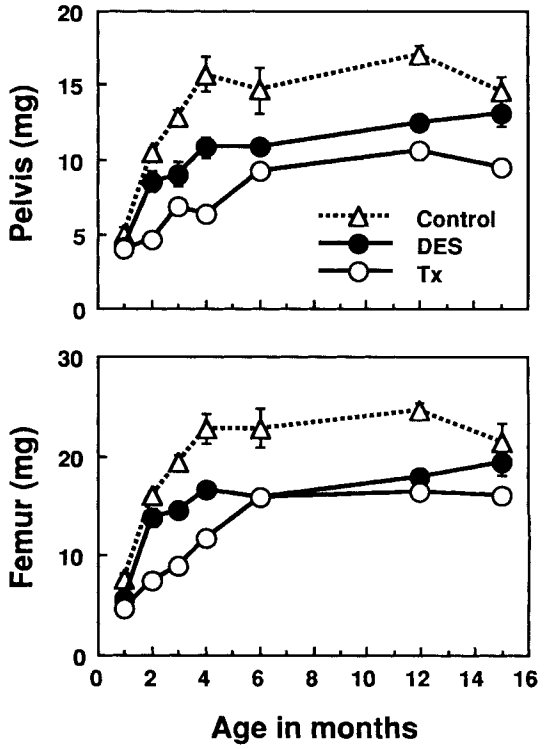

Fig. 3. Changes in ash weight of pelvis (**upper**) and femur (**bottom**) in control and neonatally DES- and Tx-treated male mice.

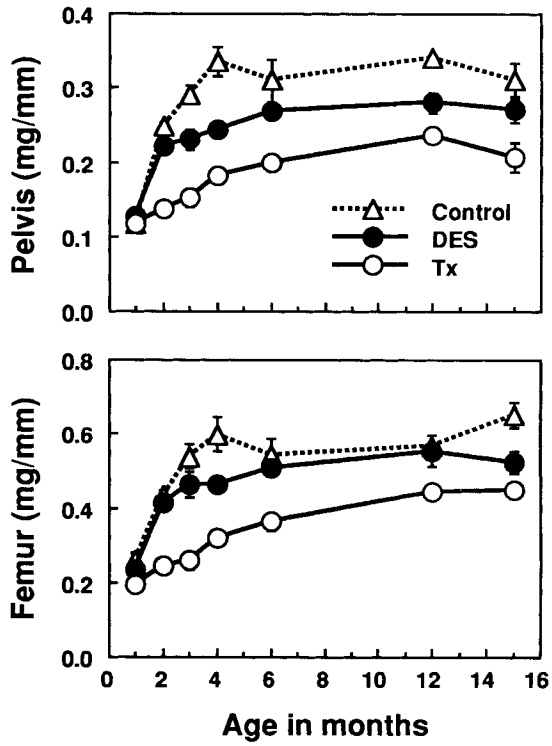

Fig. 4. Amounts of calcium per unit length (1 mm) of pelvis (**upper**) and femur (**bottom**) in control, neonatally DES- and Tx-treated male mice.

mur length between DES- and tamoxifen-treated mice (Fig. 2).

Ash weight of pelvis and femur in control mice reached a plateau at 4 months of age (Fig. 3). Ash weight of pelvis in DES- and tamoxifen-treated mice was lower than in the controls except at 1 and 15 months; ash weight of femur in DES- and tamoxifen-treated mice was lower than in the controls. The weight of femur in tamoxifen-treated mice was smaller than in DES-treated mice except at 1, 6, and 12 months of age.

Mineral Contents of Bones and Plasma

Ca and P contents of the bones were normalized to bone length and expressed per unit length (1 mm) of bones as described by Migliaccio et al. (1992). In control mice, Ca and P contents in pelvis and femur reached a plateau at 4 months of age (Figs. 4, 5). Ca content of pelvis of neonatally DES-treated mice was lower than that of the age-matched controls except at 1 and 15 months; the content of femur was lower only at 4 and 15 months. In neonatally tamoxifen-treated mice, Ca contents of pelvis and femur were lower than those of control and DES-treated mice. P contents of pelvis and femur of DES-exposed mice were lower than those of controls at all ages except at 3 and 6 months for the pelvis and 2, 6, and 12 months for the femur. P values for pelvis and femur of tamoxifen-treated mice were lower than those of controls and DES-exposed mice except for the pelvis at 9 months. There was no difference among mineral contents of either bone when expressed as mg/mg ash weight (data not shown). Mineral contents of plasma showed no significant change from controls in neonatally DES- and tamoxifen-treated male mice (data not shown).

Bone Histomorphometry

The development of cancellous bone in femur was delayed in DES-treated 2-month-old male mice compared with that in the controls (Fig. 6). Epiphyseal line (growth line) partially disappeared in femur of 12- (2/8) and 15-month-old (8/8) control male mice. In contrast to this, the line remained clearly in femur of neonatally DES- and tamoxifen-treated mice even at 15 months of age (Fig. 6). The number of osteoblasts per unit length (1 mm) of endocortical surface of femur was smaller in 2- and 3-month-old DES- and tamoxifen-treated male mice than in controls. However, the number of osteoblasts of femur in 12-month-old DES-treated mice was greater than in the age-matched controls (Fig. 7). The number of osteoclasts per unit length in neonatally DES-treated mice was not different from that in the controls, whereas in tamoxifen-treated mice, the value was greater than that in the controls at all ages examined (Fig. 7).

Testosterone Levels

Serum testosterone levels in control mice at 6 months of age were significantly lower than those at 3 months of age. Neonatally DES- and tamoxifen-exposed mice showed testosterone levels at both 3 and 6 months significantly lower than those of controls (Fig. 8).

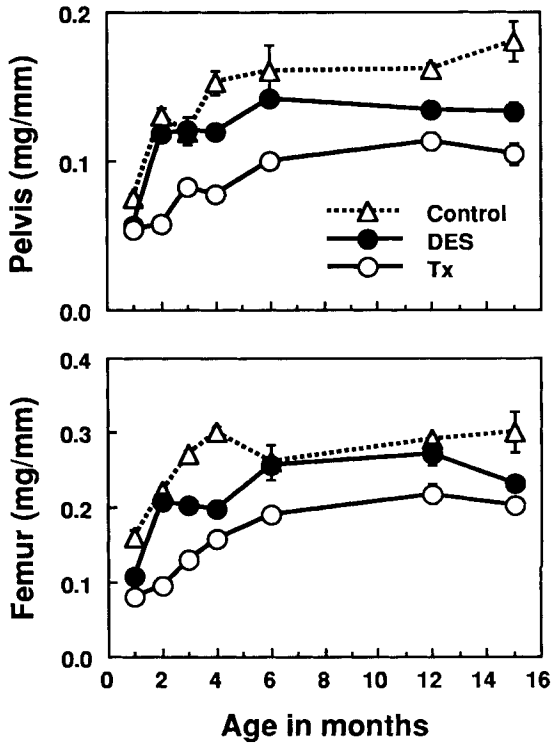

Fig. 5. Amounts of phosphorus per unit length (1 mm) of pelvis (**upper**) and femur (**bottom**) in control, neonatally DES- and Tx-treated male mice.

DISCUSSION

Perinatal exposure to DES and tamoxifen induces irreversible changes in estrogen target tissues of male and female rodents (for reviews see Takasugi, 1976, 1979; Forsberg, 1979; McLachlan, 1979; Herbst and Bern, 1981; Mori and Nagasawa, 1988; Iguchi, 1992). In the present study, we found that neonatal exposure to tamoxifen as well as to DES induces permanent changes in the pelvis and femur of male mice.

The ash weight and the length of pelvis and femur were lower in both DES- and tamoxifen-exposed male mice than in the controls. During the 30-day period from 1 month of age, the ash weight and length of pelvis and femur increased rapidly in both control and DES-treated mice. However, ash weight and length of pelvis and femur increased much less in tamoxifen-treated mice. During the 60-day period from 2 months of age, the rate increase in ash weight and length of pelvis and femur were low in DES- and tamoxifen-treated mice. Uesugi et al. (1992a,b) reported that sexual dimorphism of mouse innominate bone (pelvis) is determined by sex steroids at around 2 months of age. Serum testosterone levels in neonatally DES- and tamoxifen-treated mice at 3 and 6 months were lower than in the age-matched controls, suggesting that neonatal exposure to those compounds brings about bone loss by lowering the blood level of testosterone, since Li et al. (1995) showed that castration induces bone loss by increasing eroded perimeter and diminishing bone formation in rats. It has been reported that estro-

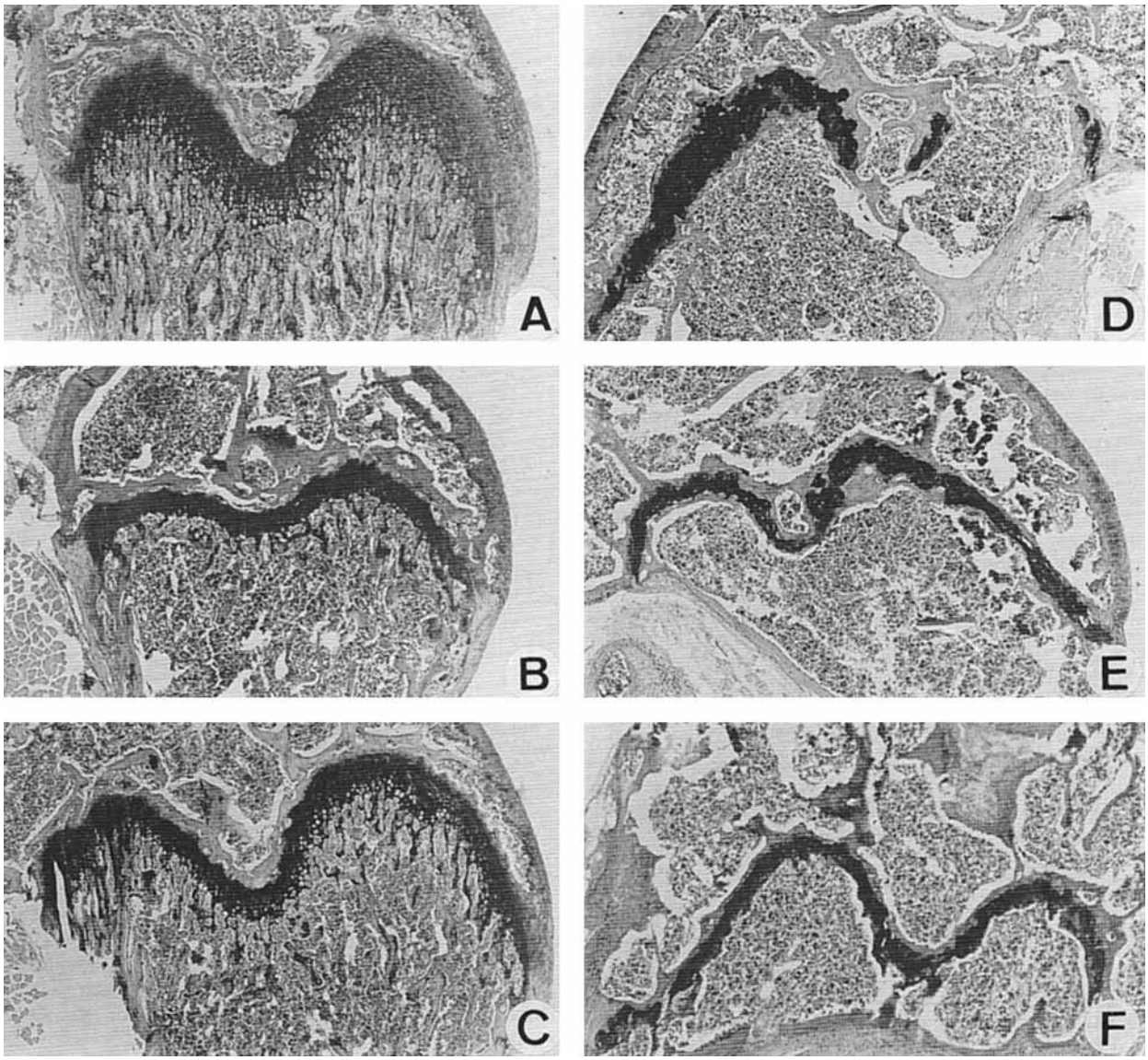

Fig. 6. Epiphysis of femur in 2-month-old control **(A)**, neonatally DES- **(B)** and Tx-treated **(C)** male mice and in 12-month-old control **9D)**, DES- **(E)**, and Tx-treated **(F)** male mice. ×55. Note the partial disappearance of epiphysial line in D.

gen and estrogen-like compound directly affect the growth plate and inhibit longitudinal bone growth (Turner et al., 1994), therefore, DES and tamoxifen might act directly on the bones. Estrogen-binding sites have been demonstrated in human and rat osteoblast-like cells (Eriksen et al., 1988; Komm et al., 1988) and in mouse periosteal cells in the pelvis (Iguchi, 1992; Uesugi et al., 1992b). Uesugi et al. (1993) showed that tamoxifen acts directly on the neonatal mouse pubis to inhibit its ossification in organ culture. Previous studies revealed that blood testosterone is aromatized to some extent, resulting in conversion of this hormone to estrogen (Ryan et al., 1972). The present results suggest, therefore, that neonatal DES and tamoxifen exposure causes the retardation of postpubertal bone growth directly and/or indirectly. Further studies are needed to determine the growth rate of longitudinal bone of mice exposed neonatally to DES and tamoxifen using double fluorescent labeling method (Hansson et al., 1972).

During bone remodeling, coupling between osteoblasts and osteoclasts involves the initial action of osteoclasts to resorb bone in a limited area. Osteoblast-derived factors that affect osteoclast recruitment and/or activity have the potential to mediate estrogen action on bone resorption (for reviews see Centrella and Canalis, 1985; Turner et al., 1994). Bone matrix-derived factors produced by osteoblasts, such as transforming growth factor-β (TGF-β) and insulin-like growth factors have been implicated as paracrine fac-

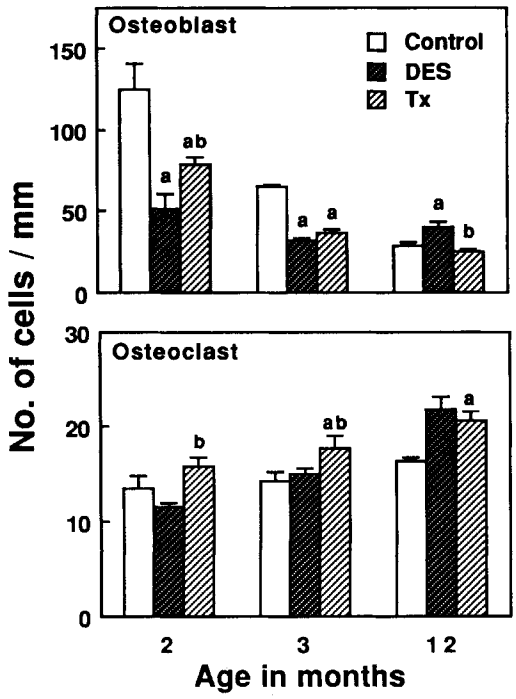

Fig. 7. Number of osteoblasts (**upper**) and osteoclasts (**bottom**) per unit length (1 mm) of endocortical surface of femur in control, neonatally DES- and Tx-treated male mice. a, $P < 0.05$ vs. control; b, $P < 0.05$ vs. DES (Student's *t*-test)

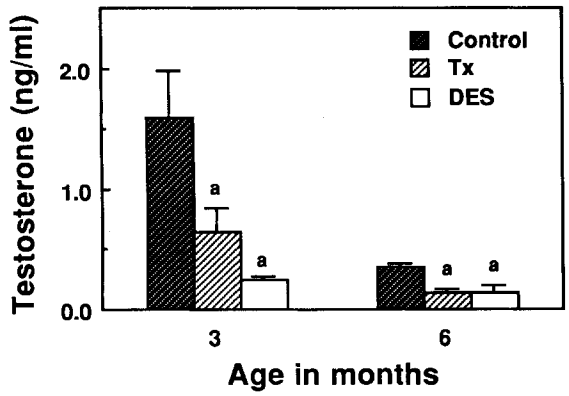

Fig. 8. Serum testosterone levels in control, neonatally DES- and Tx-treated male mice at 3 and 6 months of age. Each point consists of 5–7 samples. a, $P < 0.05$ vs. control (Student's *t*-test)

tors which might also regulate osteoclasts (Chenu et al., 1988; Pfeilschifter et al., 1988). Inhibition of bone resorption by estrogen is postulated to diminish subsequent bone resorption by preventing the release of growth factors previously deposited in the bone matrix (Turner et al., 1994). In the present study, the number of osteoblasts per unit length of endocortical surface of femur and serum testosterone levels as significantly lower *in neonatally* DES- and tamoxifen-exposed mice than those in the controls. The osteoclast number in femur in tamoxifen-exposed mice was higher than in the controls. Estrogen production in the testicular homogenates of neonatally DES-exposed mice was lower than in the controls (Ohta et al., 1995). These findings suggest that hormonal imbalance might caused the alteration in coupling of osteoblast and osteoclast as well as their numbers in femur.

A striking change in bone morphology was found in old neonatally DES- and tamoxifen-treated male mice. The epiphyseal line in the femur remained intact in male DES- and tamoxifen-treated mice even at 15 months of age, notwithstanding that this line largely disappeared in 12- and 15-month-old control mice. It is suggested, therefore, that neonatal DES exposure caused an abnormal bone turnover, resulting in retardation of bone growth in aged mice. Migliaccio et al. (1992) reported that the area of trabecular and compact bone/total bone area was significantly higher in neonatally DES-treated 12- to 14-month-old female mice than in age-matched controls. In contrast, the present study indicated that there is no difference in this ratio from the control value in the central part of the femur of 12- and 15-month-old male mice. The difference in results in the two studies may arise from the differences in the dose of DES, animal sex, and/or strains used.

Mechanical strain is an important modulator of bone remodeling (Truner et al., 1994). Unweighting the skeleton results in rapid bone loss which may be due to uncoupling of bone remodeling: bone formation is decreased and bone resorption is increased or unchanged (Whedon and Heaney, 1993). In the present study, neonatally DES- and tamoxifen-exposed mice showed that the body weight and the serum testosterone levels were significantly less than those of controls. It is reported that weightless (Westerlind et al., 1993) and ovariectomy (Ikeda et al., 1993) result in decreased TGF-β mRNA levels in rat tibia, therefore, further studies are needed to measure TGF-β mRNA levels in neonatally DES- and tamoxifen-exposed mice.

Ca and P contents in pelvis of 2-month-old neonatally DES-treated male mice were lower than in the controls, whereas in 2-month-old, DES-treated female mice, Ca and P contents were not different from those in the controls (unpublished data). The effect of neonatal DES on these bones, therefore, may differ at early stages of postnatal bone development in male and female mice. Neonatal exposure to DES and tamoxifen decreased the bone formation rate in young animals, resulting in the small-sized bones in aged mice. Tamoxifen was more effective than DES.

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