Acta Orthop Scand 55, 643–646, 1984

Forelimb malformation in rats caused by cyclophosphamide

A single dose of cyclophosphamide (20 mg/kg) was administered to female rats on Day 12 of gestation. Fetuses were collected at 24-h intervals from gestation Day 18 to 20. Gross malformations of the forelimb were observed, notably micromelia, oligodactyly, brachydactyly and adactyly. Specimens stained with Alizarin red-s and Alcian blue revealed postaxial skeletal deformities. Chronological histochemical investigations revealed delay in the ossification of the radius and completely unossified ulna; the end results showed the ulna to be more affected than the radius.

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Cyclophosphamide (Endoxan/Cytoxan/Sendoxan), interfering with the process of cell division in proliferating tissues, is teratogenic in various laboratory animals (Singh & Gupta 1972, Singh & Sanyal 1972). Limb malformation has been a common defect, most frequent in the rat when cyclophosphamide is given on Day 12 of gestation (Jeyaseelan & Singh 1984). We have now tried to identify the site of teratogenic action of cyclophosphamide in early phases of limb development in rat embryos and to correlate the possible sequelae of this with the skeletal defects of the forelimb induced by this drug.

Material and methods

Female rats of Charles Foster strain weighing about 200 g were caged overnight with males of the same strain. Pregnancy was timed by counting as Day zero, the morning on which sperms were found in the vaginal smear. A freshly prepared solution of cyclophosphamide (CPA) in normal saline was administered by a single intraperitoneal injection to pregnant rats on the 12th day of gestation (20 mg/kg).

Control rats received the same volume of saline without the drug during the corresponding gestation period. A total of 27 pregnant animals were used in the present study including six control animals. Both control and experimental animals were provided with Hind-Lever diet and tap water *ad libitum*.They were sacrificed on Days 18, 19 and 20 of gestation. Fetuses were removed by uterotomy and, after examination for any external malformations, those marked for skeletal study were fixed in 95 per cent ethyl alcohol and processed for differential staining of cartilage and bone (Inouye 1976). Representative specimens of forelimb were fixed in 10 per cent formalin and processed for histochemical examination by staining with silver nitrate (Lillie 1965) for the visualisation of calcification sites.

Results

Reduction deformities of the forelimb such as micromelia, oligodactyly, brachydactyly and adactyly were observed in all treated cases from gestation Day 18 to 20 (Figure 1). Associated anomalies included hypoplasia of the jaw, exencephaly, edema and stunted growth (Figure 1). Differential staining of cartilage and bone revealed retarded skeletal growth and suppression of various ossification centres in the ulna (Figure 2). Histochemical examination revealed delayed calcification and delayed bone marrow formation in the radius, undifferentiated cartilage cells, reduced extracellular matrix and absence of bone marrow formation in the ulna. The proliferation and growth of the cartilage cells of the ulna were more affected than in those of the radius (Figure 3).

Discussion

Cyclophosphamide-treated rat fetuses provided a convenient system for the study of

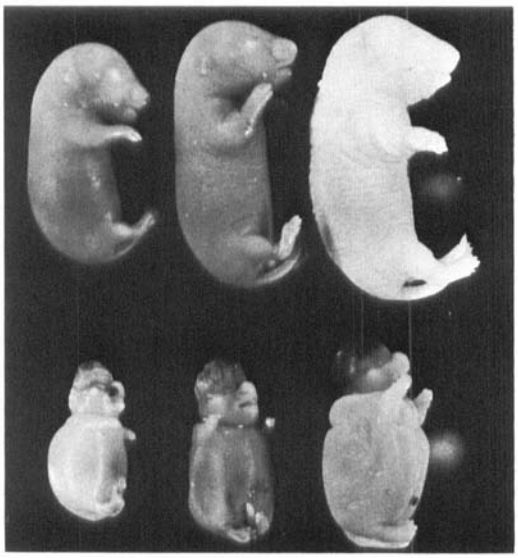

Figure 1. Cyclophosphamide-induced malformations of the forelimb of Day 18, 19 and 20 (left to right) rat fetuses. Other associated malformations are also seen. Top, corresponding controls.

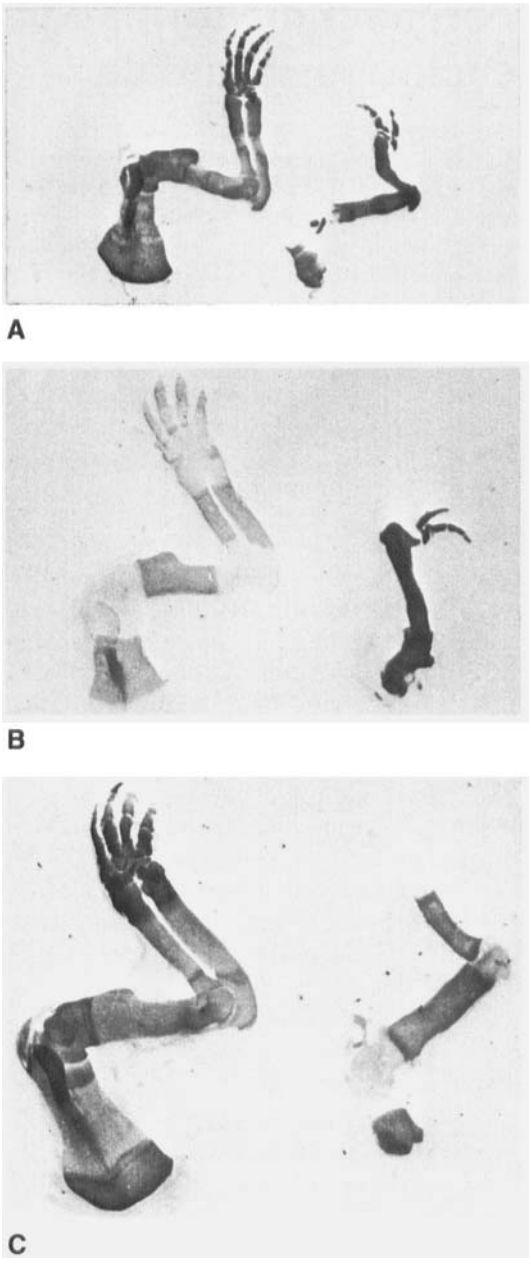

Figure 2. Cyclophosphamide-induced forelimb malformations of Day 18 (A), 19 (B) and 20 (C) rat fetuses after differential staining for cartilage and bone. Left, control; Right, treated (20 mg/kg – Day 12). Note the suppression of various ossification centres at the distal end and absence of ulna.

events relating to abnormal skeletogenesis, as they had consistent skeletal malformations. The present study has demonstrated that CPA interferes with the normal growth and differentiation of the skeletal elements of the forelimb. Since chondrification and ossification proceed in a proximodistal sequence in the limbs (Zwilling 1961), the suppressive effect of CPA depends on the stage of differentiation of the limb bud at the time of administration of the drug. CPA is known to have a selective inhibitory effect on the proliferating mesenchyme of the developing embryos (Murphy et al. 1958, Chaube et al. 1967, Singh et al. 1971) and thus interferes with normal chondrogenesis and osteogenesis. CPA is first activated in the liver of the host, resulting in the liberation of the alkylating radical (Brock 1967), the major action of which seems to be related to inhibition of DNA synthesis (Chaube et al. 1967). The prolonged inhibition of DNA synthesis may lead to localised cell death, which is sufficient to upset the proliferation rates resulting in malformations (Ritter et al. 1971). The teratogenic action of CPA has shown striking similarities in different species and even in different strains (Chaube et al. 1967, Singh et al. 1971), probably by its same mode of action. However, the brunt of the damage caused by this agent is borne mainly by the mesenchymal tissue (Hicks et al. 1957).

The postaxial skeletal deformity of the long

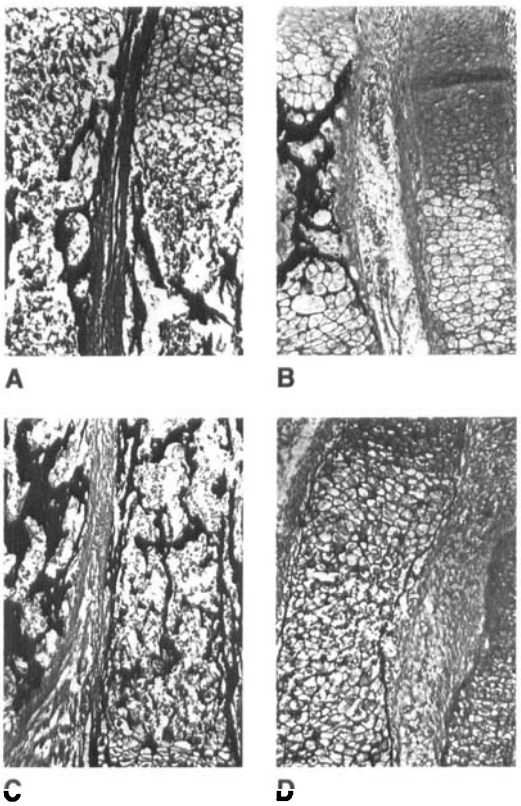

Figure 3. Photomicrograph of distal end of forelimb of Day 18 (A and B) and Day 20 (C and D) rat fetuses after cyclophosphamide (20 mg/kg – Day 12) treatment. Silver nitrate stain. ×107. A and C controls; B and D treated. Note the reduced intercellular substance, absence of calcification and marrow formation in ulna (B and D), hypertrophic chondrocytes and delayed marrow formation in radius.

bone observed in the present study is in line with the reports of Kleinebrecht et al. (1972). Inhibition of production of matrix has been considered to be responsible for inducing such skeletal deformations. Cell division, cell packing and matrix secretion determine the growth of cartilage (Wolpert 1976) and any alterations in this pattern lead to abnormal skeletogenesis. However, gravitational force (Duke 1983), the concept of positional information in terms of appropriate cytodifferentiation (Wolpert et al. 1975), and alterations in the normal morphogenetic movements of cells (Yamada 1977) should be considered for such a mechanism of limb morphogenesis.

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