Effects of cyclophosphamide on the femural epiphyseal growth plate in young Sprague-Dawley rats

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The purpose of this study was to assess the effects of cyclophosphamide on cartilaginous growth and differentiation. Cyclophosphamide is a drug commonly used in the treatment of neoplastic diseases and in preparation for bone marrow transplantation. Eighteen Sprague-Dawley rats divided into control and experimental groups received 2 i.p. injections of either saline or cyclophosphamide (30 mg/kg) with a 3-day interval starting from day 10 after birth. Effects on the proximal femoral epiphyses were evaluated histomorphometrically as well as semi-quantitatively at day 31 after birth. Results showed a significant reduction in length of the cyclophosphamide-treated femora compared to the controls. This could be attributed to a significant reduction in the thickness of the growth zone. Cell differentiation throughout the growth plates was clearly disturbed, involving nesting of cells, loss of polarity, and impaired maturation as seen by areas of excessive hyalinization. Although the effects of cyclophosphamide on the growth plates were significant compared to controls, the changes were not as extensive as previous reports have indicated. This could be attributed primarily to the fact that a comparatively low dose of the drug was used in the present study. Also, a period of recovery was allowed prior to evaluation. Nevertheless, significant effects remained which should be considered when treating young children with cyclophosphamide.
☐ *Bone; cyclophosphamide; growth; morphometry*

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Cyclophosphamide is an immunosuppressive drug used for treating neoplastic and inflammatory diseases (for a review, see 1), and in connection with bone marrow transplantation. The drug belongs to the family of alkylating agents and primarily affects the DNA synthesis and thereby cell division, cell growth, and differentiation. It acts by cross linkage to the DNA strands. However, its effects can be reversed through the action of DNA repair enzymes (2). Thus, in non-dividing cells, the alkylation of DNA by itself is not a lethal event. Although cyclophosphamide primarily affects dividing cells, it has been shown that its action is not phase specific and, consequently, it can kill cells in any phase of the cell cycle (1). The drug's toxic characteristics comprise bone marrow suppression, teratogenic and oncogenic effects. A side effect of cyclophosphamide, however, is that it promotes infections through immunosuppression.

By itself, cyclophosphamide is an inert agent which requires further enzymatic activation. Activation may occur in different organs, but it is believed that cytochrome P-450 in the liver is its main activating agent. The drug is broken down into two main metabolites, acrolein and phosphoramide mustard, both of which may contribute to the drug's effects.

The drug is administered either orally or intravenously with a wide range of dosages. The dosage is determined according to the specific medical situation. It may be given as a single agent or in combination with other drugs and/or radiation. It is further monitored individually according to total leukocyte count. However, it has been shown, in studies on long-term young survivors with acute lymphocytic leukemia, that in more than 50% of cases the dosages of cyclophosphamide diverged from the predetermined protocols by more than 25%, and in most cases the dosage was lower than the recommended one (3).

Cyclophosphamide is widely used in children for treatment of lymphoproliferative disorders such as acute lymphoblastic leukemia and in preparation for bone marrow transplantation. Acute lymphoblastic leukemia accounts for one-third of childhood malignancies in the Nordic countries (4). In these patients, side effects such as disturbed dental development and bone growth are of particular interest to pedodontists and orthodontists.

Several studies have demonstrated that cyclophosphamide can affect bone growth. Wang and Shih (5) reported that a weekly dose of 20 mg/kg in Sprague-Dawley rats resulted in a significant decrease in thickness and cellularity of the cartilage layer in the mandibular condyles. After 5 to 7 weeks, the percentage of hard tissue was significantly reduced, and the cellularity (counts per area unit of osteoblasts and osteoclasts) was reduced as well (5). Näsman et al. (6) demonstrated a reduction in the thickness of the palatal bony suture of young rats after two intraperitoneal injections of cyclophosphamide (30 mg/kg body weight) given at an interval of 3 days. Malejczyk and Moskalewski (7) reported that when repetitive high doses

of cyclophophamide (100 mg/kg) were administered to mice, degenerative changes in the transplanted allogenic epiphyseal chondrocytes were observed. Cartilage loss and bone formation were inhibited. Another consequence of this high dose of cyclophosphamide was degenerative changes within cartilage. Numerous areas devoid of chondrocytes and of basophilic material were found (7).

Abd-el-Rehim et al. (8) showed significant morphological effects of cyclophosphamide in the growth plates of mice. The recorded changes were described as a reduction in thickness and loss of palisading of the growth plate. Moreover, a reduction in the size of the cartilage cells was followed by deposition of bone adjacent to the growth plate, a condition known as epiphyseodesis or premature closure of the growth plate. However, in this study, daily doses of cyclophosphamide were administered without allowing the growing bone to recover. This may explain the relatively severe retardation seen in the epiphyseal growth.

The aim of the present study was to measure morphological effects on the femoral growth plates of Sprague-Dawley rats after a recovery period following two administrations of cyclophosphamide.

Materials and methods

Experimental animals

Three litters of Sprague-Dawley rats of both sexes with 6 siblings in each litter, in total 18 rats, were used for the study. At the age of 10 days, 12 rats from two litters received an intraperitoneal injection (0.04 ml) of 30 mg/kg body weight of Cyclophosphamide (Sendoxan®, ASTRA G, Frankfurt, Germany) dissolved in sterile water. One litter with six control rats was injected with 0.04 ml saline. At 13 days of age, a second injection of 30 mg/kg body weight of cyclophosphamide was given to the experimental group, and 0.04 ml of saline was given to the control rats. The rats were also used in a study of the effect of cyclophosphamide on the odontogenesis (9), and this is the main reason why the ages 10 and 13 days were chosen for the injections. At these ages, significant development of roots (1st molars) and crowns (3rd molars) takes place.

During the following period, all rats were weighed three times a week and their furs were examined. At the age of 31 days, all rats were sacrificed by decapitation after CO_2 asphyxiation (4 min).

Histological preparation

The dissected femora were fixed in neutral-buffered 10% formaldehyde. Three specimens (two from the experimental group and one from the control group) were excluded from the final analysis due to histo-technical problems. The specimens from both groups (10 experimental and 5 controls) were rinsed in PBS for 1 week and than dissected free. Later, the femora were measured (macroscopic measurements) and then decalcified with 20% formic acid for 1 week. The femora were trimmed into two halves and embedded in paraffin in a way which allowed sectioning (4–5 μm) in the frontal plane. The sections were stained with hematoxylin and eosin. Three sections from the widest part of the epiphysis were selected for histo-morphometric evaluation.

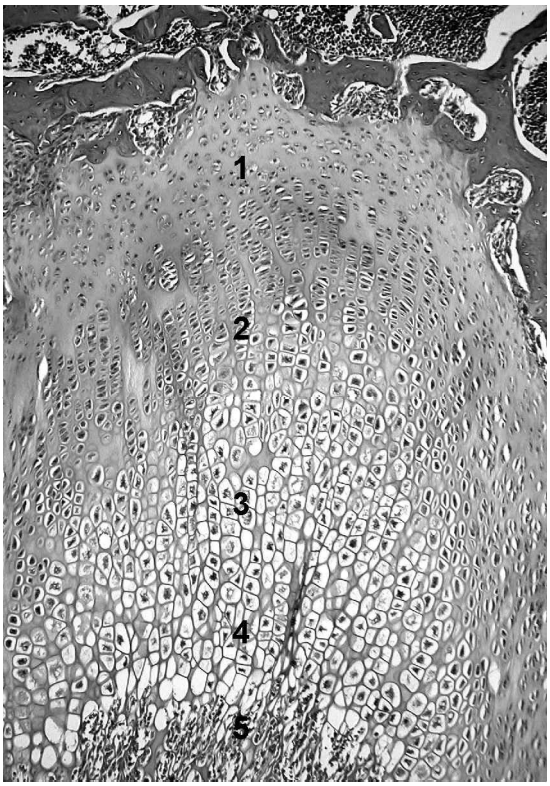

Fig. 1. Schematic division into different zones of the distal epiphyseal growth plate of the femur from a control rat age 31 days. 1. Zone of reserve cartilage. 2. Zone of proliferation and maturation. 3. Zone of hypertrophy. 4. Zone of calcified cartilage. 5. Zone of resorption. The thickness of zones 1 to 3 was measured in this study. Original magnification 125×.

Morphometric evaluation

The influence of cyclophosphamide on growth of the femora was assessed histomorphometrically as well as macroscopically. Two different measurements were obtained: 1) Macroscopic measurements of the dissected femora, both of the overall length and the maximal thickness of the sagittal aspect of the epiphysis and 2) microscopic measurements of the thickness of the first 3 layers of the growth plate.

The epiphyseal growth plate in rats, like that in humans, has a well-organized structure and can be divided into 5 different zones with specific characteristics (Fig. 1). The thicknesses of the zone of reserve cartilage, zone of proliferation and maturation as well as the zone of

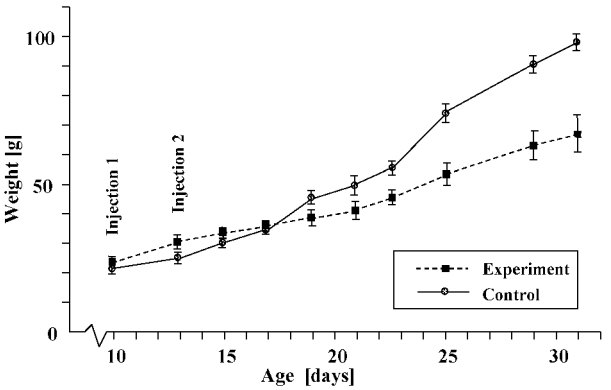

Fig. 2. Change in body weight of the experimental and control rats from 10 to 31 days of age.

hypertrophy were determined using a computer-based image analysis equipment (Hamamatsu Argus-50, Hamamatsu City, Japan).

The measurements were performed at 3 different locations across the epiphysises in the frontal plane. Measurement 1 was performed in the middle part of the plate where it is narrowest. Measurements 2 and 3 were performed in the lateral and medial segments where the thickness was greatest. The system was calibrated with a micro-scale with 10 μm divisions. The calibration procedure was performed before and after completion of the morphometric measurements.

Statistical evaluation

The Wilcoxon-Mann-Whitney U-test was used to evaluate the statistical significance of the differences between the measurements obtained from the experimental and control groups. On the basis of double determination of each variable in 20% of the total number of sections, the error of method was calculated according to the Dahlberg (10) equation ($S_i = \sqrt{\sum d^2/2N}$). The error variance was found to be less than 3% of the biologic variance for all variables.

Results

The experimental rats did not increase in weight at the same rate as the controls. During the time of observation, the weight gain of the experimental animals was linear, whereas an accelerated weight gain was observed in the controls (Fig. 2). The cyclophosphamide treatment regime, however, seemed to have a minimal effect on the physical activity of the rats. A transient alopecia on the heads and shoulders of the rats was noted. At the end of the experiment normal growth of the fur had been regained.

The femora of the cyclophosphamide-treated rats were on average 1 mm shorter compared to the femora of the controls (19.5 and 20.5 mm, respectively; $P < 0.05$). Both the zone of proliferation and maturation and the zone of

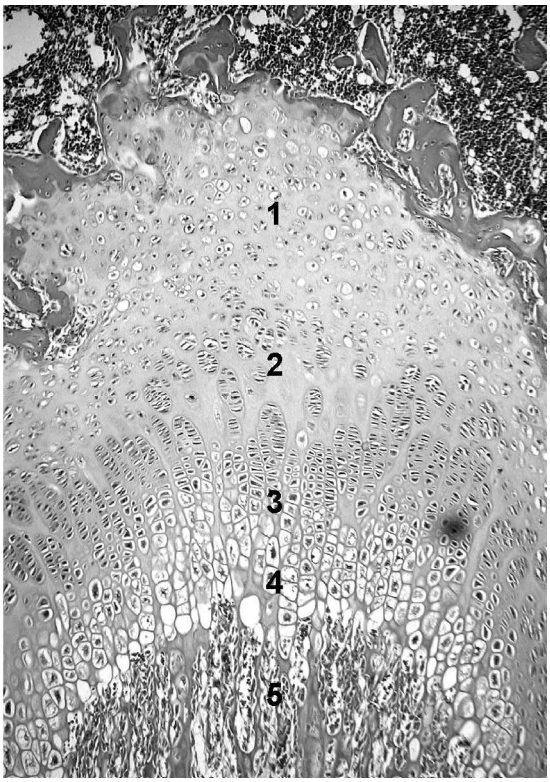

Fig. 3. Schematic division into different zones of the distal epiphyseal growth plate of the femur from an experimental rat age 31 days. 1. Zone of reserve cartilage. 2. Zone of proliferation and maturation. 3. Zone of hypertrophy. 4. Zone of calcified cartilage. 5. Zone of resorption. The thickness of zones 1 to 3 was measured in this study. Original magnification 125×.

Table 1. Mean thickness and SD (μm) of the different layers of the epiphyseal growth plate in the experimental and control groups as measured in 3 predetermined sites across the histologic sections. An asterisk denotes values which differ significantly ($P < 0.05$) between the experimental and control groups

	Experiment			Control		
	Side 1	Middle	Side 2	Side 1	Middle	Side 2
Reserve Cartilage Zone	185.1 (22.9)	94.2 (7.2)	228.4 (24.0)	168.0 (27.0)	94.3 (22.5)	174.6 (16.9)
Proliferation and Maturation Zone	323.0 (30.2)*	253.8 (20.0)*	324.2 (16.1)*	356.3 (16.1)*	289.7 (16.0)*	393.6 (24.0)*
Hypertrophy Zone	283.0 (28.4)*	194.9 (19.9)*	310.8 (49.3)*	455.2 (19.4)*	274.0 (47.3)*	505.1 (35.9)*
Total	575.4 (39.2)*	542.9 (39.6)*	863.8 (38.5)*	979.5 (42.7)*	658.0 (73.1)*	1073.8 (41.4)*

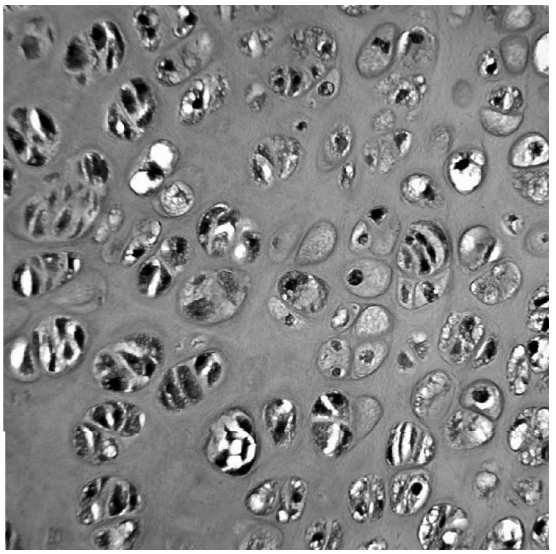

Fig. 4. Disturbed palisading of the chondrocytes in zone 2 in an experimental rat age 31 days. Original magnification 310×.

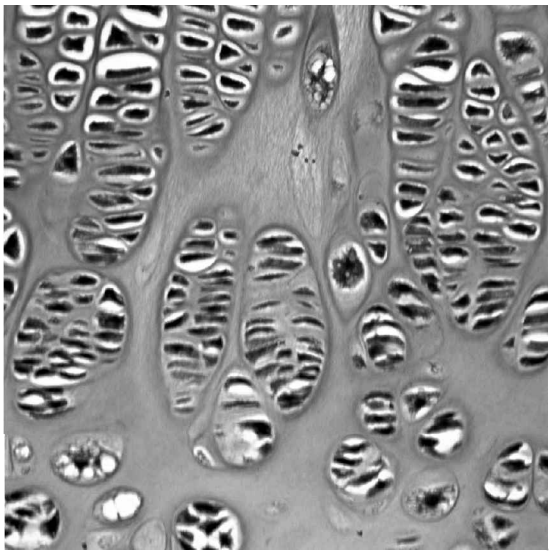

Fig. 6. Nesting or clustering and acellular zones within the zone of proliferation and maturation (zone 2) in an experimental rat age 31 days. Original magnification 310×.

hypertrophy were significantly reduced in thickness in all locations in the experimental group in comparison to the control group ($P < 0.05$). The thickness of the reserve cartilage layer, however, did not differ significantly between the groups (Table 1). In addition, the overall total thickness of the 3 layers was reduced in all locations in the experimental group in comparison to the control group ($P < 0.05$). This was also confirmed when combining the medial-lateral measurements along the growth plate.

The control animals displayed a regular and uniform arrangement of the growth plates (Fig. 1). The histological analysis showed that the growth plates in the experimental group were generally disorganized (Fig. 3) with a disturbed palisading of the chondrocytes (Fig. 4). In the reserve zone, some chondroblasts also exhibited multiple or giant nuclei (Fig. 5). Nesting or clustering of chondroblasts and acellular regions were observed consistently in the proliferation and maturation zones (Fig. 6). The cells in the zone of reserve cartilage and in the zone of proliferation and maturation also appeared to be smaller in the experimental rats than in the control rats.

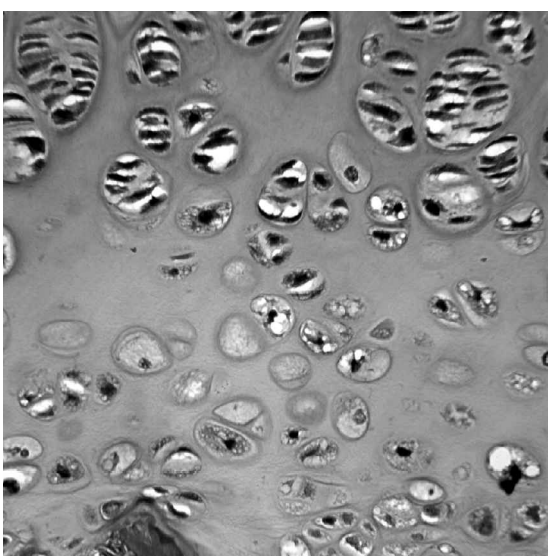

Fig. 5. Chondroblasts with multiple or giant nuclei in the reserve cartilage zone (zone 1) in an experimental rat age 31 days. Original magnification 310×.

Discussion

The epiphyseal growth plate, which is usually well defined, is the major site of longitudinal growth of long bones (11). It consists of a cartilaginous layer which, on the basis of histological morphology, can be divided into different zones. In the process of growth in this site, cartilage tissue is replaced by bone tissue. This process comprises proliferation, maturation, apoptosis, and calcification of cartilage cells, which are then resorbed and replaced by bone matrix. In the present study, femora of young Sprague-Dawley rats were used to study the epiphyseal growth after 2 intraperitoneal injections of cyclophosphamide followed by a recovery period of 18 days.

The results showed that cyclophosphamide has a retarding effect on longitudinal growth of long bones in Sprague-Dawley rats. In comparison with the control femora, the femora from the experimental group were 1 mm shorter on average, and a significant reduction in the thickness of the epiphyseal plate was also recorded in this group.

Qualitative disturbances in the different zones, such as

loss of palisading, atypical cells along the different layers, chondroblasts with giant nuclei, nesting or clustering of cells, differences in cell size and mitotic figures in the reserve cartilage zone were also observed. These effects of the drug are in agreement with the results from Abd-El-Rehim and co-workers (8), although the effects on bone growth in that investigation seemed to be much more pronounced. The difference may be attributed to the fact that a larger dose was administered distributed as daily injections for 30 days, whereas administration of the drug in this study was limited to 2 doses with a 3-day interval.

As indicated by the present results, the inhibitory effects on bone growth from cyclophosphamide appear at least partially reversible, as indicated by the lack of effect on the thickness of the reserve cartilage. This observation is partly supported by Bar-On and co-workers (12), who studied the effect of different chemotherapuetic agents, including cyclophosphamide, on the human distal femural growth plate in a small group of young patients treated for osteosarcoma. In their material, no reduction in the total thickness of the growth plate was observed, but mild signs of disturbance in orientation of the columnar cell arrangement of the growth plate were seen. Their finding that premature closure of the growth plate does not occur is in agreement with the present result, but is in disagreement with Abd-El-Rehim and co-workers (8).

The finding of Bar-On and co-workers (12) of a lower cell count along the columns in the proliferating part of the growth plate can be expected to result in a reduction in the thickness of the corresponding growth zone. This is in agreement with our results of a reduced thickness of the proliferation and maturation zone. However, no such correlation appears to be at hand for the hypertrophy zone. It is therefore tempting to speculate that under optimal clinical conditions no premature closure of the growth plate will occur.

The present findings are further supported by human studies (13–15), where growth has been found to be disturbed in children treated for malignancies in general and leukemia in particular. In those studies, reduced longitudinal growth, as defined by failure to obtain a predicted target height, was significant in the chemotherapy treated children, and was even more pronounced when therapy included radiation therapy (13, 14). However, the findings reported by Herber et al. (15), that a significant catch-up growth may occur in long-term survivors of childhood malignancies treated by chemotherapy alone, are in agreement with the results of our study.

After administration of two 30 mg/kg doses of cyclophosphamide with a 3-day interval followed by a recovery period of 18 days, a reduction in the histomorphometric thickness of all epiphyseal zones except the reserve cartilage zone was found. This was accompanied by morphological evidence of disturbed differentiation and macroscopic evidence of retarded longitudinal growth. However, no signs of premature closure of the growth plate were found. These findings indicate that, although treatment with cyclophosphamide may impair bone growth, the potential to recover some of the lost growth may be retained.

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