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EFFECTS OF CYCLOPHOSPHAMIDE ON MECHANICAL PROPERTIES OF BONE AND SKIN IN RATS

HENRIK WIE, LARS B. ENGESAETER & EVA I. BECK

Institute for Surgical Research, Rikshopitalet, University of Oslo, Norway

The effects of several different doses of cyclophosphamide on weight increase, longitudinal bone growth and mechanical properties of bone, intact skin and skin wounds, were studied in 30 young, male rats. The cytostatic effect was evaluated by counting white blood cells (WBC) in arterial blood at the end of the medication period.

Compared with the control animals, the longitudinal bone growth, the mechanical properties of the distal femoral metaphysis and the skin wounds were most noticeably affected by the drug. Diaphyseal torsional strength of the femoral bone and tensile strength of intact skin were less affected.

Key words: bones; cyclophosphamide; mechanical properties; rats; skin; tensile strength; white blood cells; wound healing

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Cyclophosphamide has been found to retard the healing of open, granulating skin wounds in rats (Wie et al. 1979) and to reduce the tensile strength of incisional skin wounds in mice at doses comparable to intensive therapeutic dose schedules in man (Cohen et al. 1975).

Interference with the development of bone tissue in rats by damaging the cartilage cells of the epiphyseal plate of the long bones has been observed by Giambelli (1961). The drug also delays reabsorption of bone fragments and re-formation of bone in rats and rabbits with experimental fractures (Fesani et al. 1964, Mangione & Taverna 1964).

To our knowledge, the effects of cyclophosphamide on the mechanical properties of growing bones have not previously been investigated. The present study in rats, therefore, was undertaken to assess the effects of various doses of cyclophosphamide on the mechanical properties of growing bones, intact skin and sutured skin wounds. The weight increase of the rats and the longitudinal bone growth were also evaluated.

MATERIALS AND METHODS

Thirty male, inbred WKY/N/Mol (F 21), SPF rats aged 24 days, with a mean initial weight of 75.3 ± 11.3 g, were used. Five to six animals were kept in each cage. Standard animal pellets (Bl.nr. 3155, Møllesentralen, Oslo) and water *ad libitum* were supplied. The animals were kept in rooms with automatic regulation of light, temperature and relative humidity. The animals were weighed every second day. An additional six rats were sacrificed immediately to provide initial bone length values. For the experiments the rats were divided into two weight-matched groups, one was given cyclophosphamide, the other placebo.

Cyclophosphamide (Sendoxan®, Pharmacia, Uppsala, Sweden) was dissolved in sterile water and injected intraperitoneally every second day for 14 days, the first injection being given immediately after infliction of the wounds. Controls received corresponding injections of isotonic vehicle. The cytostatic effect of the medication was evaluated

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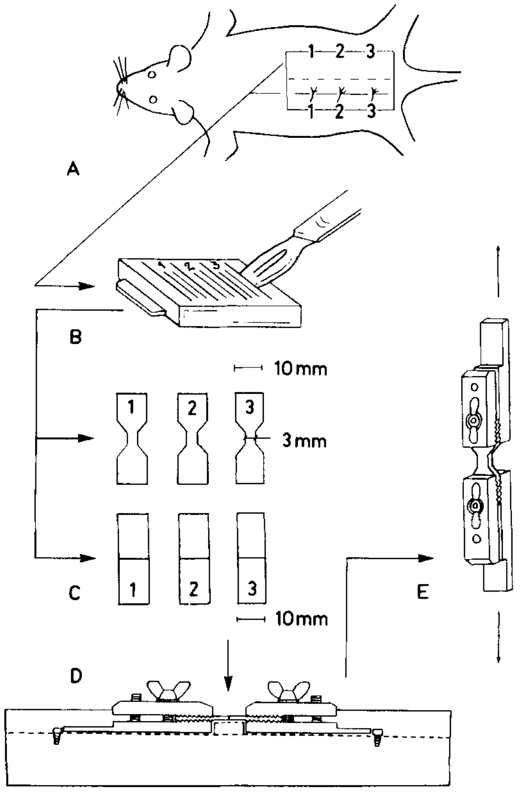

Figure 1. Schematic drawing of the equipment used for preparation and tensile strength testing of intact and sutured skin specimens. (A) rat with specimen sites, (B) template for preparation of specimens, (C) skin specimens, (D) mounting equipment, (E) clamps for mounting in the tensile testing machine.

by counting white blood cells (WBC) at the end of the medication period.

After intramuscular anaesthesia (Hypnorm®, Mekos) the backs of the animals were shaved and cleansed with 0.5 per cent chlorhexidine. Incisional skin wounds were inflicted under asceptic conditions using the following procedure:

One centimeter to the left and parallel to the caudal part of the spine a 3.5 cm longitudinal incision was made through the skin and down to the fascial layer (Figure 1A). The wounds were closed with three interrupted sutures (4–0 Vicryl®, Ethicon) and left undressed. The sutures were removed after 1 week. No signs of infection were seen in the wounds.

Fourteen days after the start of the experiments the rats were anaesthetized with ether and blood was collected by puncture of the aorta at the iliac bifurcation. Death followed by exsanguination. Immediately after death the femurs were dissected free from surrounding tissues and stored frozen (−20° C) until tested.

After thawing in isotonic salt solution the specimens were tested within 2 hours. The length of the right femur was measured from the top of the caput to the distal end of the medial condyle with a sliding caliper (reading accuracy 0.01 mm).

The bending strength of the distal right femoral metaphysis was measured 3 mm proximal to the epiphyseal plate as described by Engesaeter et al. (1978).

The left femur was subjected to diaphyseal torsional tests: the distal end of the femur was attached to a rotating disc by means of a specially designed clamp and tested by outward rotation of the distal part relative to the proximal part (Engesaeter et al. 1978).

Samples of wounded skin were removed from the left side of the backs of the animals and intact skin from the right side. The skin was frozen in Petri dishes at −20° C in a naturally stretched condition until tensile testing was performed. By means of a special template (Figure 1B) the skin samples were cut with a scalpel, while still frozen, into appropriate specimens. The template is provided with four main parallel clefts, 30 mm long and 10 mm apart. Between these clefts there are two smaller clefts, 7 mm long and 3 mm apart. The wounded skin was cut into three 10 mm wide strips with the wound in the centre. The three intact skin specimens were prepared with a 3 mm wide central narrowing to prevent rupture at the clamps when tested (Figure 1C). Three strips were obtained from each side of the animal. The clamps and the mounting equipment (Figure 1D, E) ensured a constant separation of 10 mm between the clamps.

Both bending and torsional tests of the femoral bones, and the tensile testing of skin samples were performed in a standard, electro-hydraulic tensile strength tester (Type 7–1/1, A/B Lorentzen & Wettre, Stockholm, Sweden) which was run at a constant rate (2.5 degrees per second for bone tests, and 0.125 mm/mm/s for skin). The load values were transferred by a transducer (HBM Kraftaufnehmer Type U 1, load range 0–10 Kp, Hottinger Baldwin Messtechnik, Darmstadt, W. Germany) to a chart recorder (Riken Denshi Co. Ltd., Tokyo, Japan, model SP-J 5 B).

Statistical analyses

Medians with 25- and 75- fractiles were used to express the average and dispersion of the measured values. Statistical significance was determined using the Wilcoxon two-sample test and differences were considered significant if $P \leqslant 0.05$ (Diem & Lentner 1975).

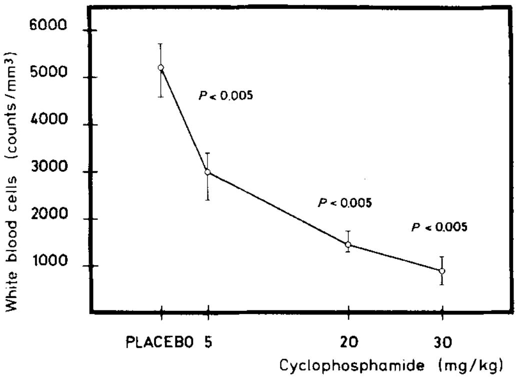

Figure 2. Median white blood cell counts (WBC) in the arterial blood of the rats after 5, 20 and 30 mg/kg body weight of cyclophosphamide and placebo given intraperitoneally every second day for 14 days. Twenty-five- and 75-fractiles are indicated. P$\leqslant$*0.05: significantly different from controls.*

RESULTS

After 14 days of medication the WBC count per mm^3 had dropped to 60 per cent in the rats on the 5 mg/kg dose schedule, to 30 per cent with the 20 mg/kg dose schedule, and to 20 per cent with the 30 mg/kg dose schedule, as compared with the placebo group (Figure 2).

The weight increases of the animals showed a corresponding course except for those on the lowest dose schedule. These animals exhibited a significantly greater weight increase than the placebo group ($P=0.05$). The weight gain in the 20 mg/kg group was about 30 per cent lower and in the 30 mg/kg group about 73 per cent lower than in the placebo group (Figure 3).

The median initial length of the right femur was 22.18 mm (Figure 4). The longitudinal growth of the femurs was affected by the 20 and 30 mg/kg schedules, with values about 91 and 89 per cent, respectively, of control ($P<0.005$). The lowest dose schedule (5 mg/kg) showed a reduction of approximately 1 per cent compared with control values ($P=0.05$). Based on the initial bone length measurements, longitudinal bone accretion showed a reduction of 6, 69.5 and 83 per cent, respectively, for the 5, 20 and 30 mg/kg schedules when compared with bone accretion in the control animals.

The metaphyseal bending moment of the right femurs showed a reduction of about 13 per cent ($P<0.005$) for the 5 mg/kg schedule when compared with the placebo group (Figure 5). In the highest dosage groups, however, the reduction was about 35 per cent ($P<0.005$).

Diaphyseal torsional tests of the left femur did not give as clear-cut results as the bending test of the metaphysis (Figure 5). There was a percentage reduction of 6 to 10 for the 5 and 20 mg/kg schedules, but of only 1 to 2 per cent for the 30 mg/kg schedule. Only the reduction in the 20 mg/kg schedule was, however, significant ($P=0.025$).

The mechanical properties of intact skin of growing rats was not significantly influenced by the 20 mg/kg schedule. The 5 and 30 mg/kg schedules, however, resulted in about a 20 per cent reduction in the strength ($P=0.05$ and $P<0.005$, respectively) of intact skin (Figure 6).

Sutured skin showed a linear relationship between tensile strength and dose level (Figure 6) with reductions of about 11, 24, and 37 per cent for the 5, 20, and 30 mg/kg

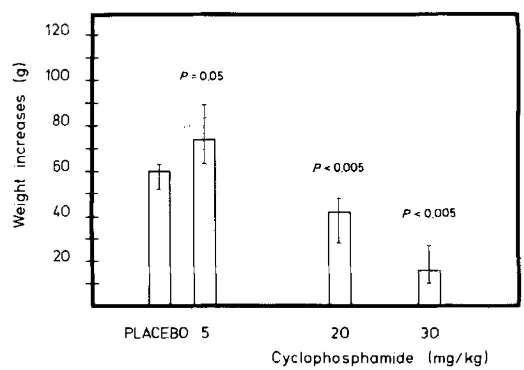

Figure 3. Effect of various dose schedules of cyclophosphamide on the body weight development in rats. Columns show median weight increments with 25- and 75-fractiles indicated. Cyclophosphamide and placebo were given intraperitoneally every second day for 14 days. P$\leqslant$*0.05: significantly different from controls.*

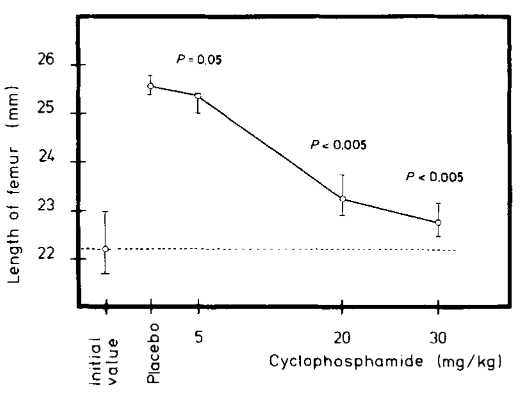

Figure 4. Length of the right femur measured from the top of the caput to the distal end of the medial condyle. Cyclophosphamide doses and placebo were given intraperitoneally every second day for 14 days. Median values with 25- and 75-fractiles are indicated. Dotted line indicates initial length measurements. P⩽*0.05: significantly different from controls.*

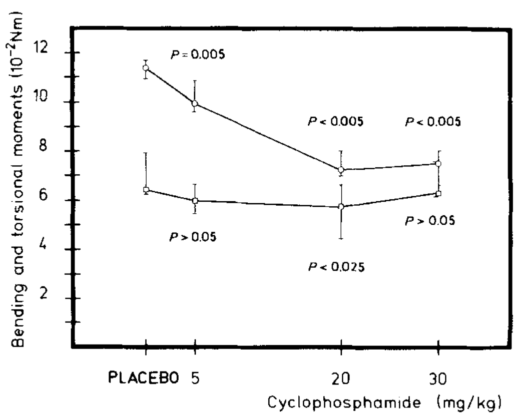

Figure 5. The bending moment of the distal right femoral metaphysis 3 mm proximal to the epiphyseal plate (○–○) and the torsional moment of the left femur diaphysis (□–□). Various doses of cyclophosphamide and placebo were given intraperitoneally every second day for 14 days. Median values with 25- and 75-fractiles are indicated. Nm = Newtom meter. P⩽*0.05: significantly different from controls.*

schedules, respectively. The effect of the lowest dose schedule was, however, not significant.

DISCUSSION

The present study has revealed that the longitudinal growth of bone and the development of mechanical strength of bone and skin in young rats are affected by cyclophosphamide. These effects seem to be dose dependent, and were observed even with a total dose of 35 mg/kg (5 mg/kg every second day for 14 days), which gave a 40 per cent reduction in the WBC count. With doses giving a WBC count reduction of 70 to 80 per cent of normal, the metaphyseal bending strength of the right femur was reduced by about 35 per cent compared with the controls, and the longitudinal bone accretion by 70 to 80 per cent.

The weight development of the rats did not follow the expected pattern as there was a seemingly increased growth rate in the 5 mg/kg schedule as compared with the controls. There seems to be no reasonable explanation for this observation.

Torsional tests of the left femoral diaphysis did not reveal clear-cut differences between placebo and the various dose levels of the

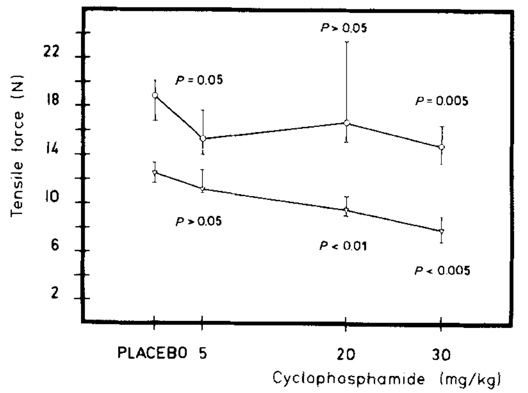

Figure 6. Tensile force necessary to pull apart intact (○–○) and wounded (△–△) skin specimens from rats given various doses of cyclophosphamide or placebo intraperitoneally every second day for 14 days. Median values with 25- and 75-fractiles are indicated. N = newtons. P⩽*0.05: significantly different from controls.*

drug. The reason may be that the torsional test is not as sensitive to drug effects in bone as is the bending test, which yielded significant differences at all dose levels. The observation may, however, reveal real differences in the susceptibility of the diaphyseal and metaphyseal bone to the drug. Some of the explanation may be found in the mode of growth of the femur (Engesaeter & Skar 1978). During the medication period only a small part of the cortex of the diaphyseal bone is formed, whereas the longitudinal growth, which is mainly confined to the epiphyseal plate near the knee, was found to be 3 to 4 mm in control animals. In the 5 mg/kg schedule nearly all the tested metaphyseal bone (3 mm proximal to the epiphyseal plate) was produced under the influence of the drug and this may explain the difference between metaphyseal and diaphyseal test results. The explanation is not, however, valid for the 20 and 30 mg/kg schedules which reduced median bone accretion to 1.1 and 0.6 mm, respectively, during the observation period.

The tensile strength of intact skin was reduced 12 to 20 per cent by the dose levels used in the present study as compared with placebo controls, and skin wound strength was reduced by 11 to 37 per cent at the same dose levels. As far as wound tensile strength is concerned, the present results are in agreement with the findings of DesPrez & Kiehn (1960). Also the observations of Calnan & Davies (1965) agree with those of the present study as they found no significant effect on wound tensile strength after a total dose of 35 mg/kg spread over 5 days. The effect of elevated dose levels on the tensile strength of incisional wounds are in agreement with the results of Cohen et al. (1975).

There is a possibility that the highest dose levels in the present study produce a cachectic condition in the animals, which might explain the marked influence on longitudinal bone growth and bending strength of the femurs. The fact that a significant influence on these parameters was observed at the lowest dose level (5 mg/kg × 7), at which the weight increase of the animals was not adversely affected, and where the WBC count was kept at a tolerable level, indicates that the effect is not merely a manifestation of generalized toxicity, but may result from a direct influence of the alkylating agent on the tissue components participating in the formation of bone. The observation of a concomitant influence on bone and skin tissues supports the assumption (Cohen et al. 1975, Hansen & Lorentzen 1975) that the main common denominator of these tissues, the collagen framework, is affected by the drug.

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Correspondence to: H. Wie, Dental Faculty, University of Oslo, Geitmyrveien 71, Oslo 4, Norway.