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EFFECTS OF OXYTETRACYCLINE ON MINERALIZATION OF BONE IN YOUNG RATS

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The metabolism of minerals and collagen in young rats receiving oxytetracycline was studied by employing double-isotope techniques. The dosage of the antibiotic was adjusted to obtain plasma concentrations comparable with human therapeutic levels. Reduced mineralization and possibly increased resorption of bone were observed after oxytetracycline administration, whereas no effect on the rate of collagen synthesis could be detected.

Key words: calcification; calcium; collagen; ¹⁴C-proline; phosphorus; plasma concentration; tetracyclines; strontium-85

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We have previously shown that administration of the antibiotic oxytetracycline to young rats causes reduced mechanical strength of bone and skin (Engesaeter & Skar 1978) and possibly interferes with the cross-linking of collagen (Engesaeter et al. 1980). Several in vitro studies (Kaitila 1971, West & Storey 1972) have indicated that tetracyclines exert their main effect on bone by inhibiting the calcification. In vivo studies, however, have provided confusing results (Bevelander et al. 1960, Ogawa et al. 1961, Harris et al. 1968, Gudmundson 1971).

The present study was undertaken to further elucidate the in vivo effect of oxytetracycline on calcification of bone. The rate of mineralization was assayed by incorporation of strontium-85 into bones (Cohn & Gusmano 1967) and the rate of collagen synthesis by measuring ¹⁴C-hydroxyproline derived from ¹⁴C-proline (Firschein 1969). These isotope measurements were supported by determinations of the content of calcium, phosphorus and collagen in the bones.

MATERIALS AND METHODS

Fifty outbred male Wistar/Af/Han/Mol SPF rats were used. At the start of the experiment the animals were 23 days old, weighing 33–37 g. They were kept five in each cage and fed water and Norwegian standard diet for rats (containing 0.9 per cent calcium and 0.7 per cent phosphorus) ad libitum. The rats were divided into two weight-matched groups of 20 animals, one oxytetracycline treated and the other control. Ten rats were killed at the beginning of the experiment to obtain origin values of bone size and composition.

The treated animals received 2.8 mg oxytetracycline (Terramycin® Intravenous, Pfizer) in 0.5 ml water as intraperitoneal injections every twelfth hour for 7 days. As the rats grew rapidly the dose in mg/kg/day was higher initially than at the end; in the middle of the period 74 mg/kg was given twice a day. The control rats received corresponding injections of the vehicle. Plasma concentrations of oxytetracycline were determined on the fifth day of medication by the paperdisc method of AB-biodisk (Stockholm, Sweden) (Jalling et al. 1972).

Twenty-four hours before the rats were killed 5 μCi carrier free strontium-85 chloride
(330 Ci/mmol) and 15 μCi L-(U'4C)proline (285 mCi/mmol) (The Radiochemical Centre, Amersham) per 100 g body weight were injected intraperitoneally.

On the third and the seventh day of the medication 10 rats from each group were anaesthetized with ether and blood was collected by puncture of the aorta at the iliac bifurcation. The animals were then killed by exsanguination. Both femurs were immediately dissected free and the length measured (from the top of caput to the distal end of the medial condyle) with a sliding callipers (accuracy of ± 0.01 mm). Soft tissues, cartilages and epiphyses were removed. Both femora were then placed in acetone for 7 days with two changes of acetone and air dried at 35°C until constant weight (dry weight) was obtained. The bones were then hydrolyzed in 4 ml 6M HCl at 125°C for 18 hours.

As hydroxyproline is practically unique to collagen, quantitation of collagen was performed by measuring the content of hydroxyproline in the hydrolysates (Firschein 1969). The rate of collagen synthesis was assayed by the conversion of 14C-proline to 14C-hydroxyproline (Firschein 1969). Mineralization rate was assessed by measuring the content of 85Sr (Firschein 1969). The bone hydrolysates, containing both 85Sr and 14C, were first counted in a well counter (Auto-Gamma Scintillation Spectrometer, Packard Instr. Comp.) to determine the content of 85Sr (gamma-emitter), without interference from 14C (beta-emitter) (Firschein 1969). The counting efficiency for 85Sr was found to be 26 per cent. Dowex 50W columns were used to separate 14C-hydroxyproline from 14C-proline, as described by Firschein (1969). In addition, these columns also retain 85Sr (Firschein 1969). Every sample was, however, checked in the Auto-Gamma Scintillation Spectrometer to make sure no gamma-emitter was present, before the radioactivity of the isolated 14C-hydroxyproline was assessed in a liquid scintillation counter (Nuclear Chicago, Mark II) with Dilsolvent® (Packard Instr. Corp.) as scintillation solution. Counting efficiency, determined by the two channel ratio method, was 70 per cent.

The concentrations of calcium and phosphorus in the hydrolysates were measured using a Bichromatic Analyzer (Abbott Laboratories, Diagnostic Division, USA). The Bichromatic Analyzer was also applied to measure serum concentrations of calcium, phosphorus and albumin. The kits used in the analyses were from the Abbott Laboratories (A-Gent Albumin and Calcium) and the Phosphorus Auto/Stat kit of Pierce Chemical Co. (Rockford, Illinois, USA).

The median with 25- and 75-fractiles was used to express the average and the dispersion of the measured values. Statistical significance was evaluated by the Wilcoxon test for two samples, and differences were considered significant if $P \leq 0.05$ (Diem & Lentner 1975).

RESULTS

On the fifth day of medication the plasma concentrations of oxytetracycline were measured. Two hours after administration of oxytetracycline the plasma concentration was 15 (14–16) μg/ml, 6 hours after administration it was 2.6 (2.3–3.0) μg/ml, and 12 hours after administration less than 1 μg/ml.

The increase in the body weights of the antibiotic-treated rats was significantly less than that of the controls (Figure 1). Figure 2 illustrates longitudinal growth of the right femur. The bones of the animals receiving oxytetracycline were significantly shorter than those of controls on both the third and the seventh day of medication, 1 and 5 per cent, respectively. Corresponding reductions were revealed in the cortex thickness and in the diameters in the middle of the femur diaphyses. At the end of the experiment the dry weight of the femurs was found to be 20

![Figure 1. Body weight of the oxytetracycline-treated and the control rats (median with 25- and 75-fractiles).](image-url)
per cent less in the oxytetracycline-treated rats than in the controls (Figure 2).

The biochemical analyses revealed corresponding differences. On the seventh day of medication the content of calcium and phosphorus in the bones of antibiotic-treated animals was significantly reduced (22 and 23 per cent, respectively) compared with controls, while the reduction in collagen content was 10 per cent. No significant differences could, however, be detected on the third day. To express the degree of mineralization of the bones, the content of calcium and phosphorus was related to the content of hydroxyproline (collagen) (Figure 3). A reduced calcium/collagen and phosphodcollagen ratio were found in the bones of the antibiotic treated rats. The differences between the two groups were 5 per cent for calcium and 8 per cent for phosphorus on the third day of medication, and on the seventh day 11 per cent for calcium and 10 per cent for phosphorus.

The content of $^{85}$Sr in the femurs related to the collagen content, the specific activity of $^{85}$Sr, was significantly less for the rats receiving oxytetracycline than for the controls on the seventh, but not on the third day of medication ($P=0.06$) (Table 1). No differences in the specific activity of $^{14}$C-hydroxyproline were found (Table 1). The relationship between these specific activity values, the ratio of the specific activity of $^{85}$Sr to the specific activity of $^{14}$C-hydroxyproline,
Table 1. Specific activity of strontium-85 and \(^{14}\)C-hydroxyproline, and the ratio of specific activity of \(^{85}\)Sr to \(^{14}\)C-hydroxyproline in the femurs of oxytetracycline-treated and control rats. Four to ten animals in each group. Data given as median with 25- and 75-fractiles

<table>
<thead>
<tr>
<th>Day</th>
<th>Sp.act. of (^{85})Sr (dpm/nmol hyp)</th>
<th>Sp.act. of (^{14})C-hyp (dpm/nmol hyp)</th>
<th>Sp.act. (^{85})Sr/Sp.act. (^{14})C-hyp (dpm/dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td>Control</td>
<td>Oxytetracycline</td>
</tr>
<tr>
<td>3rd</td>
<td>13.46 (13.33–14.56)</td>
<td>NS</td>
<td>15.42 (14.66–17.06)</td>
</tr>
<tr>
<td>7th</td>
<td>13.39 (12.42–14.00)</td>
<td>(P&lt;0.05)</td>
<td>14.24 (13.58–14.95)</td>
</tr>
</tbody>
</table>

NS = Not significant

Table 2. Concentration of calcium, phosphorus and albumin in serum of oxytetracycline-treated and control rats. Ten animals in each group. Data given as median with 25- and 75-fractiles

<table>
<thead>
<tr>
<th>Day</th>
<th>Calcium (mmol/l)</th>
<th>Phosphorus (mmol/l)</th>
<th>Albumin (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td>Control</td>
<td>Oxytetracycline</td>
</tr>
<tr>
<td>3rd</td>
<td>2.75 (2.67–2.85)</td>
<td>NS</td>
<td>2.78 (2.70–2.84)</td>
</tr>
<tr>
<td>7th</td>
<td>2.83 (2.72–2.90)</td>
<td>(P&lt;0.05)</td>
<td>2.66 (2.59–2.72)</td>
</tr>
</tbody>
</table>

NS = Not significant
called the accretion ratio (Firschein 1969), is also shown in Table 1. The accretion ratio was significantly lower in bone of oxytetracycline-treated rats than in controls on both the third and the seventh day of medication.

The concentrations of calcium, phosphorus and albumin in serum are given in Table 2. Compared with the controls, the antibiotic-treated animals had significantly higher serum levels of phosphorus on the third day (16 per cent) and of calcium on the seventh day of medication (6 per cent).

**DISCUSSION**

The main features of this study are that bones of rats receiving oxytetracycline had reduced size and reduced content of calcium, phosphorus and collagen when compared with controls. Furthermore, the antibiotic was found to affect the accretion of strontium-85, but not the accretion of $^{14}$C-hydroxyproline.

Plasma concentrations of oxytetracycline in this experiment were comparable with human therapeutic levels (Otten et al. 1975). To obtain these concentrations, however, the dose had to be about five times higher than recommended in humans (30 mg/kg/day) (Goodman & Gilman 1975).

The demonstrated reduction in body weight of the oxytetracycline-treated animals is in accordance with earlier experience (Engesaeter & Skar 1978). This observation cannot be explained by the effects on skeletal weight. Other possibilities have to be considered, such as a general inhibition of protein synthesis as described by Yeh & Shils (1966), although no inhibition of albumin or collagen synthesis by oxytetracycline has been demonstrated in this or in a previous study (Engesaeter et al. 1980). An alternative explanation could be that this broad spectrum antibiotic may have caused such a substantial disturbance of the normal symbiosis between the rat and the intestinal microorganisms (Gustafsson 1971) that it would interfere with the growth of the animal.

$^{14}$C-proline and strontium-85 are convenient isotopes to use in simultaneous studies of collagen and mineral dynamics (Firschein 1969). Although the general reservations about such isotope techniques have to be taken into consideration in the interpretation of the results (Laitinen 1967, Firschein 1969), the content of $^{14}$C-hydroxyproline and $^{88}$Sr in the bones indicates the rate of collagen synthesis and of mineralization during the 24-hour period.

Judging by the specific activity of $^{14}$C-hydroxyproline, collagen synthesis was not inhibited by the antibiotic. The content of collagen in the bones of the oxytetracycline-treated rats compared with the controls was, however, significantly less on the seventh day of medication. These data might be explained by an increased resorption of collagen in the treated rats. This assumption might also be supported by our previous finding that oxytetracycline may interfere with the cross-linking of collagen (Engesaeter et al. 1980). Collagen with defective cross-links has been shown to be more susceptible to degradation than normal collagen (Harris & Farrell 1972).

Enhanced resorption of bone cannot, however, explain the observed reduction in the accretion ratio ($^{88}$Sr/$^{14}$C-hydroxyproline) and the reduced content of strontium-85, calcium and phosphorus in bones of oxytetracycline-treated rats. The only reasonable interpretation of these findings seems to be inhibition of the mineralization process by the antibiotic, either directly or indirectly.

The mechanism by which tetracyclines interfere with the mineralization process is poorly understood. Bevelander et al. (1960) concluded that "the inhibition of mineralization following injection of tetracycline is probably due to the reduction in the number of free cations which subsequently results in the formation of a bone deficient in minerals". One mole of oxytetracycline may form complexes with 1–2 moles of calcium, depending on the relative concentrations
(Ibsen & Urist 1962). The amount of calcium bound to the antibiotic at an oxytetracycline concentration of 10 µg/ml would, however, represent only about 1 per cent of the total calcium in serum. It seems, therefore, difficult to explain in this way the negative effect of tetracycline on the mineralization, as has also been stated by Sternberg (1966) and Shapiro et al. (1977).

In vitro experiments have indicated that tetracyclines inhibit bone mineralization by preventing the transformation of amorphous calcium phosphate to crystalline apatite (Wadkins et al. 1974). Tetracyclines have also been suggested to induce defects of calcification by inhibiting the accumulation of calcium in the mitochondria of cells preparing for mineralization (Shapiro et al. 1977). This intramitochondrial accumulation of calcium is postulated by Lehninger (1970) to be an essential first step in the calcification process. Shapiro et al. (1977) found in their tetracycline experiments with chickens a profound drop in the intramitochondrial calcium concentrations and, as also observed in the present study, an increase in the serum calcium level.

Tetracyclines could, however, be considered to interfere with the calcification process in an indirect manner. It has been proposed that the formation of intermolecular cross-links in collagen may be a prerequisite for appropriate mineralization of bone matrix (Avioli 1973, Rosenquist et al. 1977). Our previous finding that oxytetracycline may impair the cross-linking of collagen (Engesaeter et al. 1980) might, therefore, indicate that the reduced mineralization is secondary to an induced defect in the collagen framework.

REFERENCES


EFFECTS OF OXYTETRACYCLINE ON MINERALIZATION


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