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Inhibitory Effect of Salmon Calcitonin on Bone Resorption: Morphological Study of the Tibial Growth Plate in Rats

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Summary. Salmon calcitonin (sCT) at doses of 100 and 50 UI given subcutaneously to growing rats produced *in vivo* evidence of osteoclastic activity inhibition. Histological assessment was carried out by measuring the perichondrial ring of Lacroix height, and a dose-correlated effect was found. These aspects were coupled with an increase in the osteoclast number and suggested that in studies with bone resorption inhibitors, morphological evaluation based on osteoclasts count is not reliable. The changes of the metaphysis suggested also that sCT affects the activity of hypertrophic chondrocytes of the growth plate. Plasma calcium levels did not differ significantly between treated rats and controls; an increased phosphatemia was observed in sCT-treated animals.

Key words: Bone resorption – Osteoclasts – Calcitonin – Growth plate.

The capacity of calcitonin to inhibit bone resorption has been demonstrated with organ culture techniques [1–4] and in experiments with isolated osteoclasts cultured on calcified substrates [5–7]; on the other hand, there are few histological evidences of such an effect in experimental animals [8], although morphological changes of osteoclasts have been observed with electron microscopy [9, 10]. The reported significant decrease in number of osteoclasts following administration of calcitonin [1, 11, 12] is not necessarily related to the level of resorption activity; on the contrary, other potent inhibitors of bone resorption, such as diphosphonates, increase the number of osteoclasts, but they are not active [13]. The tibial growth plate cartilage and the related perichondrial ring of Lacroix [14, 15] are characterized by fast modeling, therefore, they are a suitable model for the study of early changes of osteoclasts morphology and activity. No effects of calcitonin were observed employing histological techniques in rats, which received current therapeutic doses [8]. In this study, the experiment was carried out with high doses of salmon calcitonin to investigate the effectiveness of the hormone in inhibiting bone resorption in the fast modeling metaphysis of growing rats. The conical shape of the metaphysis is the result of high rate modeling of the perichondrial ring and metaphyseal primary trabeculae, and thus an inhibitory effect on bone resorption should soon become apparent as a modification of the morphology of the metaphysis [16].

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Table 1. Mean plasmatic levels of calcium and phosphates in rats treated with sCT (100 and 50 U daily) for 21 days and controls

Group		Plasma calcium (mg/100 ml ± SD)	Plasma phosphate (mg/100 ml ± SD)
A (CT 100 U)	n = 10	10.61 ± 0.33	9.65 ± 0.64[a]
B (CT 50 U)	n = 10	10.09 ± 0.76	10.18 ± 0.85[b]
C (Controls)	n = 10	10.34 ± 0.45	8.70 ± 0.49

Mean of group A and B were compared with C using Student's *t* test
[a] $P < 0.002$; [b] $P < 0.001$

Materials and Methods

Thirty Sprague-Dawley rats (Stefano Morini, S. Polo d'Enza, Reggio Emilia, Italy) weighing about 150 g were used, randomly distributed in three groups of 10 animals each. The rats in group A received 100 U of sCT daily (Sandoz S.p.A., Milano, Italy) subcutaneously and those in group B received 50 U of sCT daily: the 10 control rats of group C were treated daily with the same volume of the vehicle alone for 21 days.

Rats were housed three to a cage with free access to food and water. They were killed with an ether overdose after 21 days. The interval between the sacrifice and the last administration of sCT was 8 hours. About 30 cc of blood was drawn from the heart with a heparinized syringe; the samples were centrifuged and the plasma was stored in a freezer at −30°C until analyzed.

Plasma levels of calcium and phosphates were assessed with the method of o-cresolphthalein-complexon in alkaline solution [17], plasma phosphate with the direct phosphomolybdate reaction without deproteinization [18].

The right and left tibia of each animal were carefully dissected from soft tissues without damaging periosteum and perichondrium; the left tibia was fixed in neutral formalin (10%). Eight specimens were decalcified with EDTA, cut in the middle, coronal plane of the metaphysis and embedded in paraffin. Sections were stained with hematoxylin-eosin. The remaining two specimens were embedded undecalcified in Technovit resin (Kulzer and Co., Gmgh, Wehrheim, FRG), and sections of the middle coronal plane of the metaphysis were prepared with a cutting-grinding machine (Exact Apparatebau, Norderstadt, FRG). Sections of 25-μm thickness were stained by the Von-Kossa method (counterstained with neutral red). The number of osteoclasts was determined in a median coronal section of the proximal tibia of each rat, including the whole epiphysis, metaphysis, and proximal diaphysis. Only multinuclear cells in contact with a resorption lacunae on the bone surface were identified as osteoclasts. The height of the perichondrial ring was measured under the microscope with a graduated reticulum.

Right tibias were freed of periosteum and all soft tissues, stored for 7 days in an ethanol-methanol solution (50:50) at 27°C to extract lipids and water, and then dried in an oven at 100°C for 48 hours. They were then weighed and the lengths were measured with a caliber.

Table 2. Mean dry weight and length of tibia in rats treated with sCT (100 and 50 U daily) and controls for 21 days

Group		Tibias weight (g ± SD)	Tibias length (mm ± SD)
A (CT 100 U)	n = 10	0.361 ± 0.41	34.6 ± 0.3
B (CT 50 U)	n = 10	0.345 ± 0.035	34.8 ± 0.7
C (Controls)	n = 10	0.340 ± 0.033	35.2 ± 1.2

Mean of group A and B were compared with C using Student's *t* test

Observations

Treatment was well tolerated by the rats: no animals died during the experiment, and the behavior and weight gain of the animals in groups A and B were normal. Eight hours after the last administration of sCT there were no significant differences in plasma calcium levels between treated animals and controls; mean plasma phosphate was significantly higher in both treated groups compared with controls (Table 1).

The difference in tibial mean dry weight and length between rats

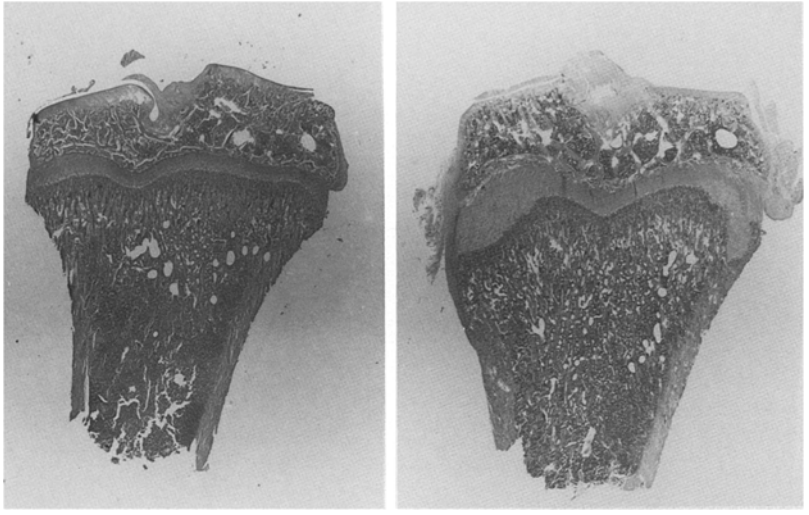

Fig. 1. Left: proximal tibial metaphysis of control rats. Right: proximal tibial metaphysis of rats treated with sCT 100 U daily for 21 days. Thickening of the outer part of the growth plate is evident as well as the cylindrical shape of one side of the metaphysis, due to failure of remodeling. Original magnification ×3.3, hematoxylin-eosin.

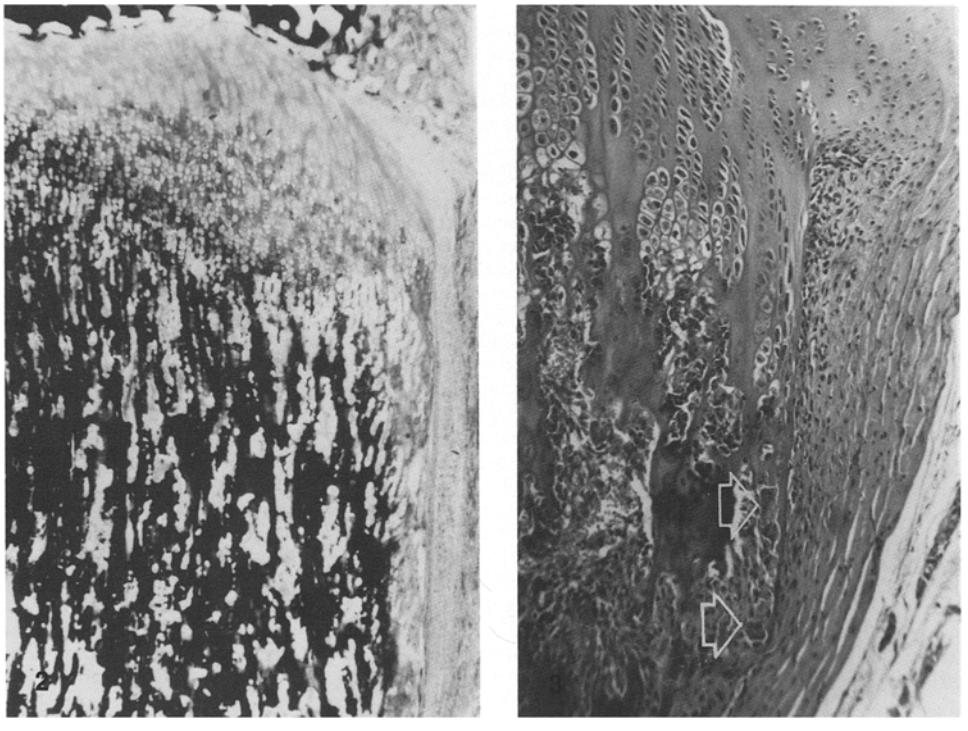

Fig. 2. Failure of calcification of intercolumnar septa of the thickened growth plate cartilage. The metaphyseal trabeculae are thicker and denser than normal, due to inhibition of remodeling. ×40, Von Kossa/neutral red.

Fig. 3. Normal aspect of the perichondrial bone bark. It does not extend below the line of primary metaphyseal trabeculae and is resorbed at its distal end by osteoclasts (arrows). ×100, hematoxylin-eosin.

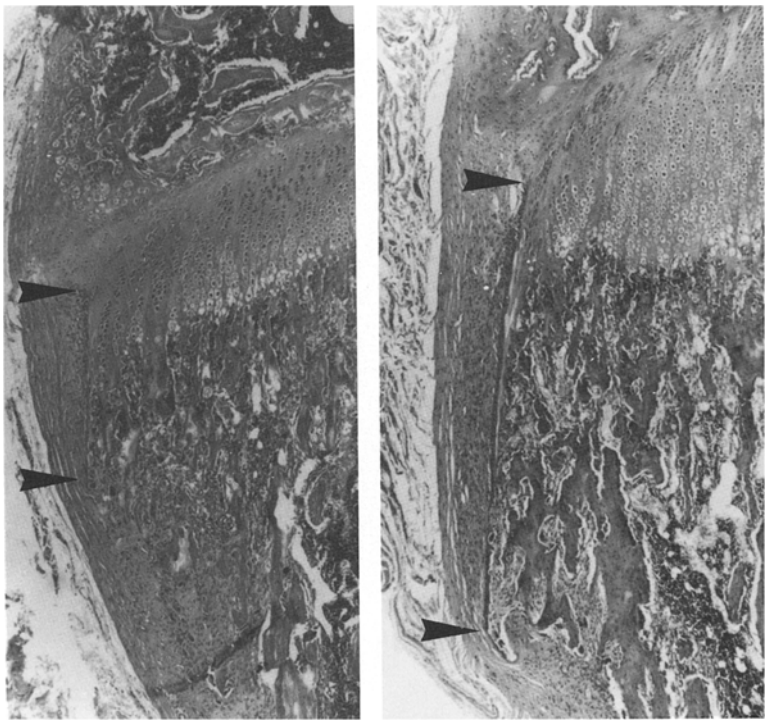

Fig. 4. The effect of sCT on the height of perichondrial bone bark (left: control, right: CT 100 U daily for 21 days). ×30, hematoxylin-eosin.

treated with sCT and controls was not significant (Table 2). The morphology of the growth plate cartilage and metaphysis in groups A and B was different from that in controls in quantity but not in quality. The growth plate cartilage showed an increased thickness of its outer part (Fig. 1). This thickening was not symmetrical and in most bones it involved only the medial side of the tibia. Thickening was due to persistence of hypertrophic chondrocytes because of failure of vascular invasion from the metaphysis. The proximal tibial metaphysis on the affected side showed an inversion of the concavity of its profile (failure of conization). Primary metaphyseal trabeculae were denser and thicker in this area (Fig. 2), as is usually observed when inhibition of resorption is coupled with a normal rate of osteoblastic apposition [25]. In the thickened growth plate failure of calcification of the intercolumnar septa was present (Fig. 2); calcification and vascular invasion were normal in the other sectors of the plate. No pathological osteoid border was present on trabeculae or cortical bone.

The perichondrial ring [14, 15, 19–21], which normally does not extend below the line of the first primary metaphyseal trabeculae (Fig. 3) and has the appearance of a very thin lamina without osteocytes, was higher in the sCT-treated animals than in controls because it lined the thickened growth plate and the unremodeled metaphysis. Also, the thickness of the ring was increased and osteocytes were present in the structure.

Because the proximal and distal ends of the perichondrial ring were easily identified on histological sections, its height could be measured (Fig. 4) and a significant difference was found when groups A and B were compared with controls (Table 3).

In the lower part of the metaphysis, a row of osteoclasts lined the bone below the periosteum. The number of osteoclasts and the ratio nuclei/osteoclast were significantly higher than in controls (Table 4). The nuclei had a dense chromatin and appeared to have an abnormally high number of picnotic nuclei (Fig. 5).

Table 3. Mean height of the perichondrial ring in rats treated with sCT (100 and 50 U daily) for 21 days and controls

Group		Height (mm ± SD)
A	n = 15	2.70 ± 1.05[a]
B	n = 12	2.21 ± 0.84[b]
C	n = 14	1.35 ± 0.32

Mean of groups A and B were compared with C using Student's *t* test
[a] $P < 0.001$; [b] $P = 0.001$

Table 4. Mean number of osteoclasts/mm^2 and mean ratio nuclei/osteoclast in rats treated with sCT (100 and 50 U daily) for 21 days and controls

Group		Number of osteoclasts/mm^2	Nuclei/Osteoclast
A	n = 10	1.14 ± 0.56[a]	5.24 ± 0.37[a]
B	n = 10	0.98 ± 0.31[a]	4.98 ± 0.39[b]
C	n = 10	0.41 ± 0.17	4.25 ± 0.67

Mean of groups A and B were compared with C using Student's *t* test
[a] $P < 0.001$; [b] $P = 0.008$

Discussion

No effects of calcitonin on the chemistry and histology of the rats' bones were observed in a previous report [8], but the dose used was small (200 mU/day). In the present experiment, 50 and 100 IU/day were used, as currently given in clinical therapy. Considering the body weight of rats, these doses are high, although comparison of the dose/body weight ratio in different species is questionable. If calcitonin is rapidly cleared from the circulation in the rat, high doses are necessary to induce the inhibitory response on bone turnover. However, the aim of this study was not to investigate the effectual doses of calcitonin in the rat, but to confirm histologically *in vivo* the effects observed in *in vitro* studies.

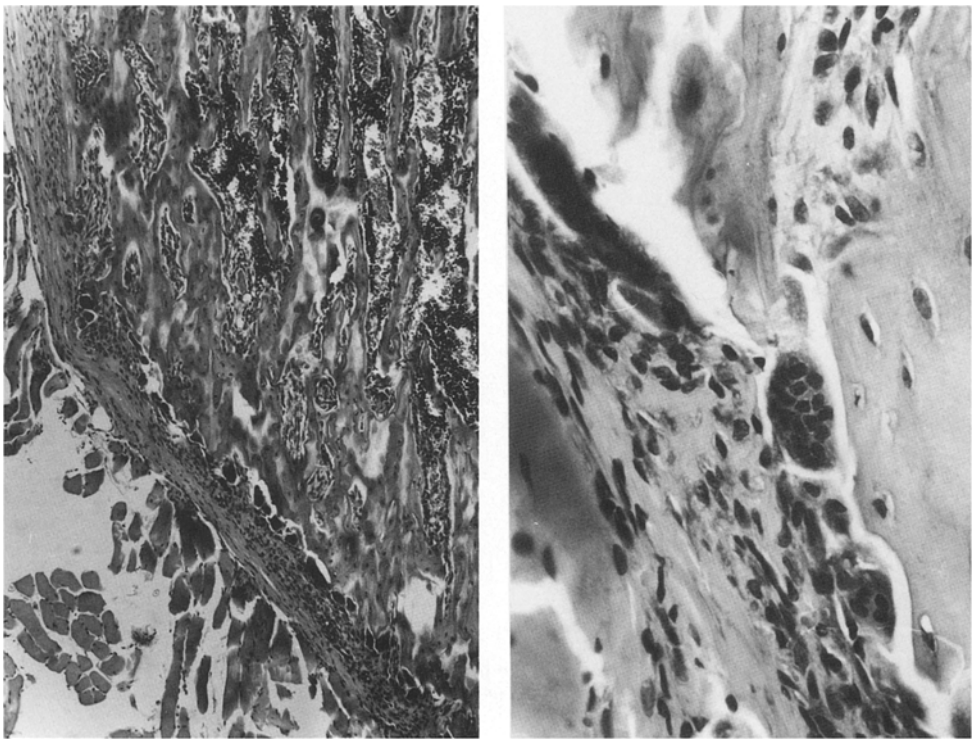

Fig. 5. Proximal tibial metaphysis of rats treated with sCT 100 U daily for 21 days. A row of osteoclasts is evident on the outer aspect of the metaphysis; they appear abnormally large and with an abnormally high number of picnotic nuclei. ×100 and ×400, hematoxilin-eosin.

Most changes of the growth plate and metaphyseal morphology observed by us can be explained in terms of inhibition of bone resorption and vascular invasion of cartilage [16]; the inhibition due to sCT involved only the outer part of the epiphyseal plate and this area was characterized by three basic phenomena: (1) inhibition of osteoclastic resorption; (2) reduced mineralization of intercolumnar septa; and (3) failure of vascular invasion.

It is difficult to explain the different response of the same anatomical structure to sCT, as well as the association of inhibition of mineralization and failure of bone resorption and vascular invasion of cartilage. The conical shape of the proximal tibial metaphysis results from a high remodeling rate, and under normal conditions, many more osteoclasts are observed on the outer border of the metaphysis than anywhere else in the bone. As osteoclasts are the major target cells of calcitonin, the inhibitory effects of the hormone obviously becomes evident earlier at sites where osteoclastic activity is higher. This could account for a different response to the hormone of different parts of the growth plate cartilage and metaphysis.

Attachment of osteoclasts to bone surface is a necessary condition for the activity of these cells both *in vitro* and *in vivo;* there are evidences that calcitonin is capable of inhibiting both mobility of osteoclasts *in vitro* and their attachment to the bone surface [6, 22]; therefore, a quantitative evaluation of *in vivo* bone resorption inhibition based on morphology is questionable. The osteoclast count has been used [11, 23], but it is not a reliable index, as the number of cells does not necessarily correlate with their degree of activity. In this study, the number of osteoclasts was observed to increase in the presence of an arrest of bone modeling.

Explanation of these observations can only be speculative: either the formation of osteoclasts is enhanced, or the life-span of inactive osteoclasts is lengthened and the cells accumulate at the resorption sites, or the large polykarios are easier identified in the histological slides, and the observed differences represent a technical artifact. On the contrary measurement of the perichondrial ring of Lacroix proved to be a simple and reliable method for assessing osteoclastic resorption inhibition and allowed comparison between groups. The inhibitory effect was proportional to the dose of the hormone (Table 3). On the other hand, there was no inhibitory effect on osteoblastic activity, as demonstrated by the thickness of perichondrial ring and primary metaphyseal trabeculae.

The sequence of phenomena that characterizes growth plate cartilage maturation suggests a correlation between cartilage mineralization and cartilage invasion by vessels. The nature of this relationship remains obscure. However, recent observations have cast doubts on the notion that chondrocytes in the lowermost hypertrophic zone are degenerating [24] and have suggested that the hypertrophic chondrocyte is a highly differentiated cell, which synthetizes and secretes into extracellular matrix a set of macromolecules, such as type × collagen, chondrocalcin, and S-100 protein, which may be directly involved in the processes of matrix mineralization and capillary invasion [25–27]. The effects on mineralization of intercolumnar septa and vascular invasion could indicate that calcitonin has an influence on the activity of hypertrophic chondrocytes.

In another study [28] in which sCT 10 IU was given for 3 and 7 days to rats weighing 20–30 g (approximately the same dose as given to group A animals in our experiment), a widening of the metatarsal growth plate cartilage was observed. Although these authors explain their findings in terms of stimulation of chondrocyte maturation, their observations confirm our results.

Calcitonin has been reported to have a hypocalcemic effect whose magnitude and duration depends on the dose [29]; 4 hours after administration of high doses of sCT (0.4 and 2 IU/100 g), hypocalcemia and secondary hyperparathyroidism have been observed [14]; the consequent increase of PTH and 1,25-dihydroxycolecalciferol secretion are known to enhance the formation of osteoclasts, while this effect is prevented by parathyroidectomy [30]. The increased number of osteoclasts and nuclei/osteoclast in the present experiment probably reflects transient changes of plasmatic calcium levels. However, as blood sampling was performed 8 hours from the last sCT administration, the animals have possibly compensated for the low calcium levels in plasma, whereas phosphate remained higher.

In conclusion, treatment with very high doses of sCT was well tolerated by rats and produced histological evidences of osteoclastic activity inhibition; these aspects were coupled with an increase of the osteoclast number and suggested that morphological evaluation based on osteoclast count is not reliable.

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