Laboratory Investigations

Morphometric Changes in the Epiphyseal Plate of the Growing and Young Adult Male Rat After Long-Term Salmon Calcitonin Administration

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Abstract. The function of the epiphyseal plate is related to the differentiation and maturation of the chondrocytes, especially of the hypertrophic zone. Salmon calcitonin exerts a positive effect on chondrocytes of different types of cartilage, e.g., articular cartilage, osteochondral callus formation, and the epiphyseal plate. In the present study, the effect of long-term daily salmon calcitonin treatment upon epiphyseal plate function was examined in 80 male Wistar rats aged 12 weeks at the beginning of the experiment. A daily dose of 6 IU of salmon calcitonin enhanced the number of the chondrocytes of the hypertrophic zone of the upper tibial epiphyseal plate, increased the mean thickness of the epiphyseal plate, and accelerated the longitudinal growth of long bones. It was found that the peripheral growth of the epiphyseal plate was delayed after calcitonin treatment in comparison with the placebo-treated animals. The most effective period for calcitonin treatment on epiphyseal plate function seems to be the late accelerated period of growth, i.e., puberty. In conclusion, long-term salmon calcitonin treatment has a beneficial effect on longitudinal skeletal growth and this effect remains throughout the adult life of the animal. Salmon calcitonin does not enlarge the surface of the epiphyseal plate.

Key words: Epiphyseal plate — Hypertrophic zone — Salmon calcitonin — Mid-term treatment — Long-term treatment — Skeletal growth — Wistar rat

Calcitonin has been widely used for decades in the treatment of several metabolic bone diseases and its clinical efficacy and safety have been thoroughly investigated [1]. In addition to the well-known antiosteoclastic effect of calcitonin [2, 3], it was also suggested that this hormone exerts a direct action on osteoblasts [4], though several other actions of calcitonin on musculoskeletal function remain unclear [5–8]. A few authors have focused their research on the action of calcitonin on the articular cartilage [9, 10] and the epiphyseal plate [6, 11]. The skeletal effects of calcitonin administration are well described [12–14]. The purpose of this study was to explore the effect of salmon calcitonin on the epiphyseal plate of normal, growing, male rats during the late pubescent period of life, the role of the duration of the treatment, and the effect of discontinuation of the administration of calcitonin on epiphyseal plate function.

Materials and Methods

Eighty male Wistar rats aged 12 weeks at the beginning of the experiment and with a mean weight of 300 g were housed under normal conditions in cages, 5 animals to each cage, and fed a standard stock diet and tap water without restriction. The animals were randomized on the basis of body weight and were allocated into 8 groups (A, B, C, D, E, F, G and H) with 10 animals in each group. All treated animals received 6 IU of salmon calcitonin subcutaneously every day, a median daily dose of calcitonin in the rat model [12]. The duration of treatment was 12 weeks (Group A treated, Group B placebo), 18 weeks (Group C treated, Group D placebo), and 24 weeks (Group E treated, Group F placebo). According to literature on this subject, an administration of salmon calcitonin to rats for a period of 18 weeks is considered a mid-term administration [13], and calcitonin treatment for up to 24 weeks is a long-term salmon calcitonin administration [14]. In Groups G (salmon calcitonin) and H (placebo), injections were stopped after 24 weeks and the animals remained untreated until the completion of 30 weeks from the initiation of the experiment. All animals were sacrificed using a high dose of anesthetic (Ketamine, Midazolam) and the left femora were dissected free of soft tissues. The dimensions of the bones (length and midshaft diameter) were measured by two independent observers using a Vernier caliper and fixed in 10% phosphate-buffered formalin, then demineralized in 10% nitric acid. The proximal half of the femur was sliced mid-sagitally, dehydrated, embedded in paraffin, and sectioned at 5 μm. All sections were stained with hematoxylin/eosin and toluidin blue. The histological quantitation of the epiphyseal plate cells was recorded by counting the number of chondrocytes in standard sites at 10 different columns of the hypertrophic zone. The width of the upper tibial epiphyseal plate and the height of the perichondrial ring were recorded as well as the presence of chondrocytes in between the normal fibroblasts of the perichondrial ring which were recorded using micrometer stage (1 mm interval 5×10^{-3}) as well as the presence of chondrocytes in between the normal fibroblasts of the perichondrial ring. Experimental procedures were reviewed and

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Table 1. Mean values and standard deviations of the measured parameters

Group	Length of femora	Number of chondrocytes	Thickness of epiphyseal plate	Width of epiphyseal plate
	Mean ± S.D	Mean ± S.D	Mean ± S.D	Mean ± S.D
A	3.73 ± 0.08	6.4 ± 0.9	4.13 ± 0.3	0.57 ± 0.04
B	3.65 ± 0.05	4.9 ± 0.3	2.7 ± 0.3	0.54 ± 0.03
C	3.80 ± 0.05	7.6 ± 0.7	3.8 ± 0.2	0.56 ± 0.03
D	3.76 ± 0.06	4.8 ± 0.6	1.75 ± 0.2	0.64 ± 0.02
E	3.83 ± 0.08	1.2 ± 0.2	2.2 ± 0.2	0.58 ± 0.04
F	3.73 ± 0.08	0.8 ± 0.1	1.5 ± 10.1	0.66 ± 0.03
G	3.84 ± 0.05	1.05 ± 0.04	2.5 ± 0.2	0.57 ± 0.05
H	3.74 ± 0.05	0.82 ± 0.03	1.5 ± 0.1	0.64 ± 0.05

The unit of the values are in mm (1 mm interval 5×10^{-3} real mm)

Table 2. Multiple pairwise differences using the Scheffe post-hoc test for the CT group during the treatment period

	A vs C	A vs E	A vs G	C vs E	C vs G	E vs G
Length of femora	0.045	0.026	0.023	N.S	N.S	N.S
No of chondrocytes per column	0.0005	0.0005	0.0005	0.0005	0.0005	N.S
Thickness of epiphyseal plate	0.008	0.0005	0.0005	0.0005	0.0005	0.023
Width of epiphyseal plate	N.S	N.S	N.S	N.S	N.S	N.S

N.S. = nonsignificant

Table 3. Multiple pairwise differences using the Scheffe post-hoc test for the placebo group during the treatment period

	B vs D	B vs F	B vs H	D vs F	D vs H	F vs H
Length of femora	0.02	0.04	0.05	N.S	N.S	N.S
No of chondrocytes per column	N.S	0.0005	0.0005	0.0005	0.0005	N.S
Thickness of epiphyseal plate	0.0005	0.0005	0.0005	N.S	N.S	N.S
Width of epiphyseal plate	0.0005	0.0005	0.0005	N.S	N.S	N.S

approved by an institutional animal care committee (3715-8/11/95) in accordance with the current National policy for experimentation on animals.

Statistical Analysis

Variables during the treatment period per group are sufficiently represented by the mean values (mean) and standard deviation (SD). Two-way analysis of variance (ANOVA) with factors of the variables treatment and time was used to evaluate the effects of drugs at different time points. We calculated the main effects and the two-way interaction between the two factors using the former model. Since interaction proved significant for variables, post hoc tests (Scheffe or LSD) were used to evaluate the differences between cell means instead of marginal means [15, 16]. We were interested to find the intergroup differences at 12, 18, 24, and 30 weeks separately as well as the within-group differences during the treatment period. The coefficient of variation of all measurements was 0.05. All tests were two-sided; $P < 0.05$ was defined as significant. All analyses were performed using the SPSS vr 8.00 statistical package.

Results

Length of Femora

All measurements and the results of the statistical evaluation are presented in Tables 1, 2, 3. In the pla-cebo-treated rats, the length of the left femur increased significantly ($P = 0.02$) during the first 6 weeks of observation (12–18 weeks) and then remained unchanged until the end of the experiment (Fig. 1). In the calcitonin-treated animals, the length of the left femur increased significantly until the 24th week of observation and then remained unchanged during the following 6 weeks (when no treatment was administered). Intergroup differences (longer femurs in the calcitonin group) were found to be significant in week 24 ($P = 0.026$) and week 30 ($0 = 0.05$) of observation. These data suggest that a long-term daily administration of salmon calcitonin to growing rats prolongs the longitudinal growth of the femur in comparison with the placebo-treated animals. This benefit remains after a 6-week discontinuation of the calcitonin injections.

Mean Number of Hypertrophic Chondrocytes

In the placebo-treated rats, the mean number of chondrocytes of the hypertrophic zone of the upper tibial epiphyseal plate remained unchanged at the completion of 18 weeks of observation and after that declined dra-
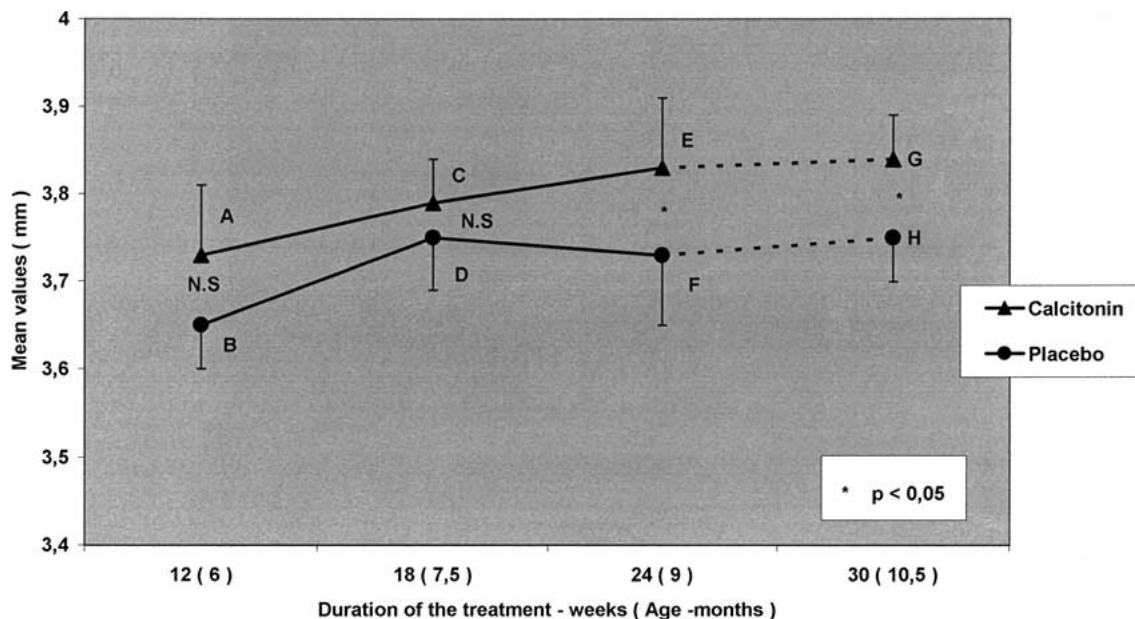
Fig. 1. Length of femora. Multiple pairwise differences using Scheffe post –hoc test between CT and placebo groups at 12, 18, 24, and 30 weeks.
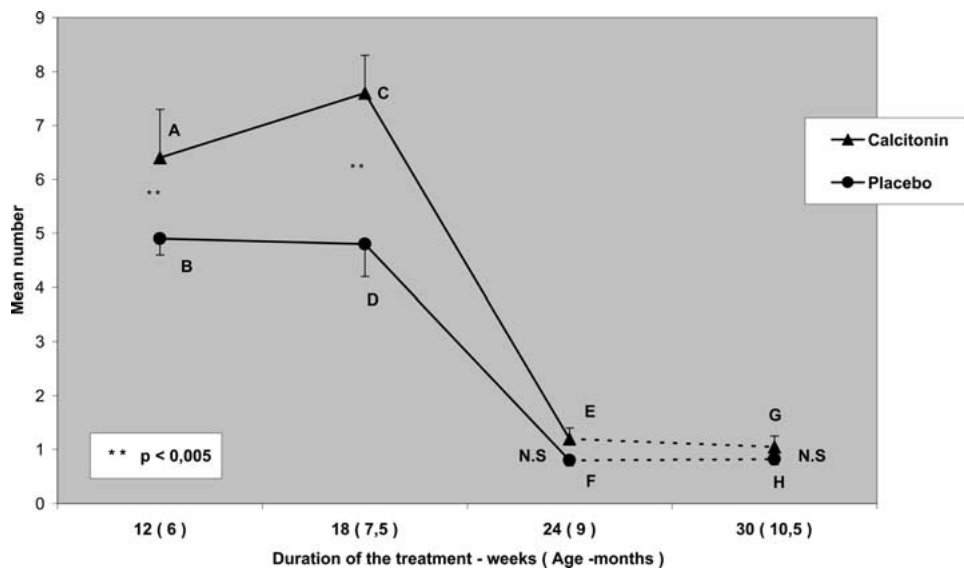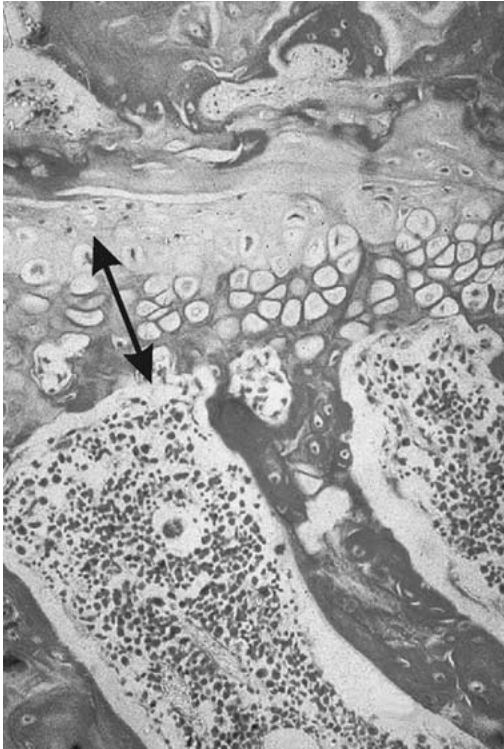

Fig. 2. Number of chondrocytes per column. Multiple pairwise differences using Scheffe post-hoc test between CT and placebo groups at 12, 18, 24 and 30 weeks.

matically ($P < 0.0005$) during the following 6 weeks and then remained unchanged until the end of the experiment (Fig. 2). On the other hand, in the calcitonin-treated animals, the number of hypertrophic chondrocytes of the upper tibial epiphyseal plate increased significantly ($P = 0.0005$) until the 18th week of observation and after that reduced dramatically ($P < 0.0005$). The inter-group differences at the 12th and 18th weeks of observation were found to be statistically high ($P < 0.0005$). At the 24th week of observation the number of hypertrophic chondrocytes in the calcitonin-treated animals was larger than in the placebo group but no statistically significant difference was found (Figs. 3,

4). This observation remained so after 6 weeks after the discontinuation of the calcitonin treatment.
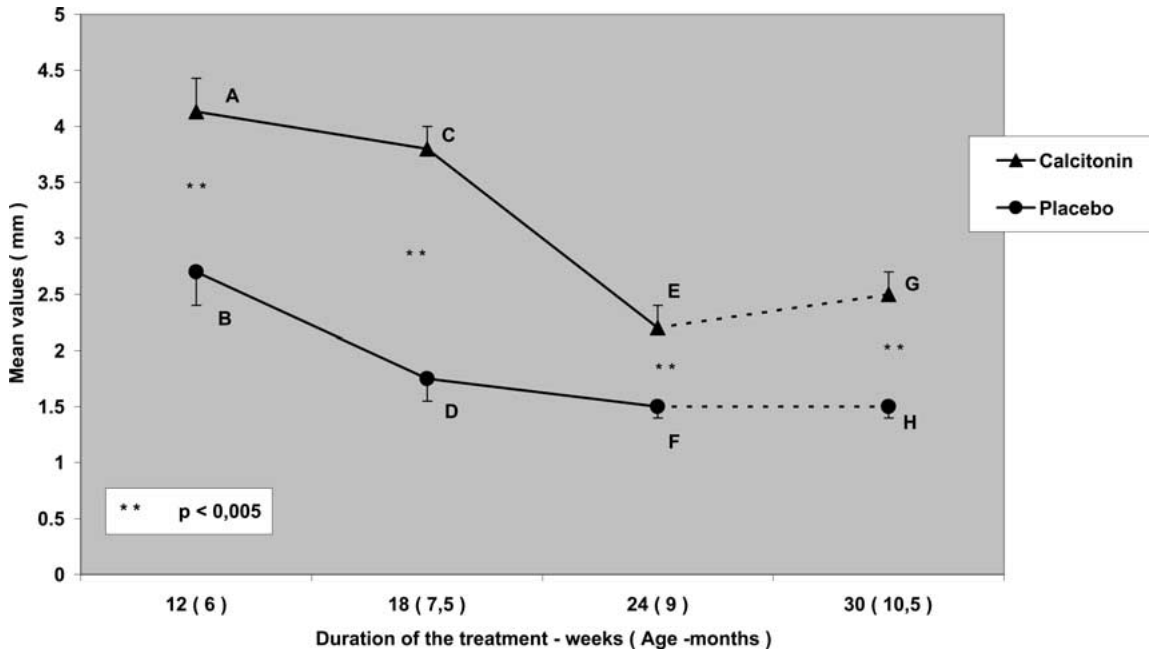
Mean Thickness of the Epiphyseal Plate

The mean thickness of the epiphyseal plate diminished significantly ($P < 0.0005$) during the treatment for both groups placebo and CT (Table 3) in relation to baseline values. Moreover, there was significant difference ($P = 0.005$) between the placebo and CT group during the whole period of treatment. After discontinuation of calcitonin treatment at the 30th week this significance remained statistically important ($P = 0.02$)

Fig. 3. A histologic section of placebo-treated rat, a thin epiphyseal plate (H/E, ×20).


Fig. 4. A calcitonin-treated animal. Notice the increase in number of hypertrophic chondrocytes and thickness of the epiphyseal plate (H/E, ×20).

Fig. 5. Thickness of epiphyseal plate. Multiple pairwise differences using Scheffe post - hoc test between CT and placebo groups at 12, 18, 24, and 30 weeks.

(Table 2). It is important to note that the thickness of the epiphyseal plate remained statistically larger throughout the experiment compared with the placebo group ($P < 0.0005$). It is speculated that there is still a tendency to retarded growth in calcitonin-treated animals as well as in other animals after discontinuation of calcitonin treatment for a period of 6 weeks (week 30) (Fig. 5).
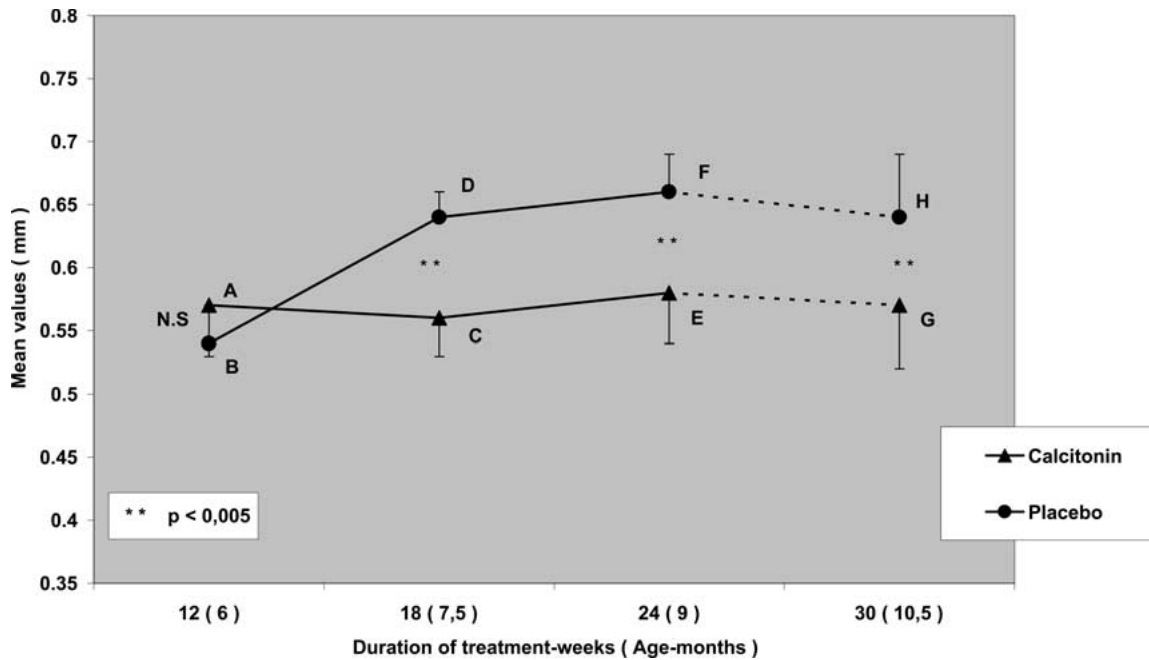
Fig. 6. Width of epiphyseal plate. Multiple pairwise differences using Scheffe post - hoc test between CT and placebo groups at 12, 18, 24, and 30 weeks.

Width of the Epiphyseal Plate and Height of the Perichondrial Ring

In the group of placebo-treated rats, the width of the upper tibial epiphyseal plate was found to increase significantly up to the age of 9 months (24 weeks of placebo treatment) ($P < 0.0005$). In the calcitonin-treated animals, the width of the epiphyseal plate remained unchanged throughout the experiment. Finally, at the end of the experiment the width of the epiphyseal plate was significantly smaller ($P = 0.002$) in the calcitonin-treated group compared with the placebo-treated animals (Fig. 6).

Discussion

Skeletal growth depends on endochondral ossification in the growth plate cartilage where proliferation of chondrocytes, matrix synthesis, and increase in chondrocyte size all contribute to the final length of a bone [17–19]. The differentiation of chondrocytes during *in vivo* processing is characterized by progressive morphological changes associated with the hypertrophy of these cells and is defined by biochemical changes that result in the mineralization of the extracellular matrix [20]. It has been suggested [20, 21] that the early hypertrophic chondrocytes have the inherent potential to differentiate to osteoblast-like cells and to contribute to initial bone formation, but only chondrocytes positioned at the borderland between cartilage and non-cartilage osteogenic tissues undergo further differentiation to bone-producing cells. He called these hypertrophic chondro-

cyte « borderline chondrocytes» to emphasize both their specific location and their dual differentiation potential. Hypertrophic chondrocytes located in different cartilage areas and exposed to an inappropriate matrix and endocrine/paracrine environment, cannot differentiate osteoblast-like cells and therefore undergo apoptosis. Gibson et al. [22] in 1995 also concluded that terminal differentiation of chondrocytes results in death by apoptotic process prior to resorption of the tissue and invasion by blood vessels. Furthermore, chondrocyte stem cells proliferate in the ossification groove of Ranvier and contribute to both peripheral and longitudinal growth of the growth plate.

Hypertrophic chondrocytes synthesize collagen type II, X, and collagenase-3 and organize their extracellular matrix, forming a tissue highly reminiscent of true cartilage which eventually mineralizes. The formation of mineralized cartilage was associated with the expression of alkaline phosphatase, arrest of cell growth, and apoptosis. Several investigators found in different studies that some hormones sped up or slowed down the proliferation-differentiation-death processing of the chondrocytes; for example, PTH/PTHrp studied by Zerga [20] showed an inhibition of both mineralization of cartilage-like matrix and apoptosis and the production of a mineralizing bone-like matrix, withdrawal of the hormonal stimulation redirects cells toward their distinct terminal differentiation and fate. Robson investigated the action of thyroid hormones on growth plate and came to the conclusion that this hormone concurrently and reciprocally regulates chondrocyte cell growth and differentiation in the endochondral growth plate [24], $1,25(OH)_2D_3$

also has an effect on the growth of the cartilage. Vit-D was detected in both proliferative and hypertrophic zones. Vit-D deficiency leads to disturbed calcification of the cartilage and enlargement of the growth plate [23].

A direct chondrogenetic effect of salmon calcitonin has been shown in previous experimental studies [6, 8–11]. Salmon calcitonin enhances cartilage growth primarily by accelerating cartilage maturation by promoting chondrocyte hypertrophy and matrix formation [6], but in newborn rats this effect seems to be temporary and is followed by an exhaustion of the epiphyseal plate [8]. In other experiments carried out on young rats, high doses (50 and 100 IU) of salmon calcitonin given for a short period (about 3 weeks) were well tolerated and without any disturbance of epiphyseal plate function [11]. In one study, long-term calcitonin administration was given to retired breeder female Wistar rats for a period of 6 months at a daily dose of 10 IU (5 days a week) and it was found that there was a reduction in ovariectomy-induced bone loss, but the effect of calcitonin on cartilage was not examined [14]. In the present experiment, a daily dose of 6 IU of salmon calcitonin per animal was considered a median dose for the rat model [12, 13]. Therefore, there is a lack of information about the long-term effect of this medication upon the epiphyseal plate, as well as about the consequences of the discontinuation of salmon calcitonin treatment.

The present study was planned to include groups of animals with a mid-term calcitonin/placebo treatment (12 and 18 weeks) as well as groups with a long-term calcitonin/placebo treatment (up to 24 weeks), and finally groups of long-term calcitonin/placebo administration (24 weeks) followed by a short-term discontinuation of the medication (an additional 6 weeks). These groups are relevant to the recommended calcitonin administration in humans [12]. It is important to notice that the experiment was planned to be performed in the late accelerated period of the growth of the rat [17, 18] and its early adult life (up to 10.5 months of life).

According to the results of the placebo-treated group, the mid-term period of treatment (12-18 weeks of observation) coincides with the later phase of the rat's skeletal growth and the extended treatment (24 and 30 weeks) coincides with a dramatic diminution of the growth plate function and the pause in skeletal growth. The effect of salmon calcitonin upon epiphyseal plate function is mainly exerted on its hypertrophic zone [6, 11] and mainly during the late phase of accelerated skeletal growth (12–18 weeks of administration). The beneficial effect of calcitonin on the epiphyseal plate remains during the early adult life of the rat (24th week of treatment) and is still present after a 6-week discontinuation of the calcitonin administration. The longitudinal growth of the placebo-treated animals (length of femora, mean number of hypertrophic zone chondrocytes, and mean epiphyseal plate thickness) ceased after the 18th week of observation

or the completion of 7.5 months of life. On the other hand, calcitonin treatment for a period of 24 weeks (rats aged 9 months) results in bigger adult animals.

This finding differs from previous studies in newborn rats [8] where the effect of salmon calcitonin upon the epiphyseal plate and metaphyseal function were found to revert after a period of treatment lasting 4 weeks. The effect of salmon calcitonin on the perichondrial ring was previously studied by Pazzaglia et al. [11]. In this study, it was found that there was a dose-related increase in the height and thickness of the perichondrial ring with the presence of osteocytes at its matrix. In the present study there were no findings to suggest that salmon calcitonin had any effect upon perichondrial ring function. On the contrary, in the calcitonin-treated animals, the diameter of the upper tibial epiphyseal plate was smaller than those in the placebo-treated animals. It is concluded that calcitonin effect is exerted mainly on the longitudinal growth of long bones and not on their circumferential growth.

Also, we would like to mention that although our study was not designed to investigate the proliferation, differentiation, and apoptosis of chondrocytes, it would be interesting as another experiment.

In conclusion, salmon calcitonin treatment (6 IU daily) for a mid-term period (6 weeks) as well as for a long-term period (12 weeks), enhances the number of cells in the hypertrophic zone of the epiphyseal plate, increases the mean thickness of the epiphyseal plate, and accelerates the longitudinal growth of long bones. The most effective period of calcitonin treatment on epiphyseal plate function is the pubescent late-accelerated period of growth. The beneficial effects of calcitonin on skeletal growth seem to remain once the animal has reached adulthood.

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