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Histological changes in the epiphyseal plate of growing young adult male rats after long-term hydrocortisone and salmon calcitonin administration

Ocena histologiczna czynności chrząstki wzrostowej szczurów poddanych długotrwałemu działaniu hydrokortyzonu i kalcytoniny łososiowej

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Abstract

Introduction: Glucocorticosteroids decrease the longitudinal growth of bones. It has been reported that skeletally immature rats on calcitonin treatment grow longer and slimmer.
The aim of this study was to assess whether concomitant treatment with calcitonin would influence longitudinal growth and histology of epiphyseal plate in hydrocortisone-treated rats.
Material and methods: Fifty six growing male Wistar rats were randomly divided into two control and four experimental groups. Rats in the first two experimental groups were treated i.p. b.i.d. with hydrocortisone, while to the other experimental animals salmon calcitonin s.c. was administered concomitantly. The controls received appropriate vehicula. The rats were kept under standardised conditions. The first phase of the experiment was completed after 28 days and right femora and tibiae were harvested from groups and preserved in 4% buffered formalin. Lengths of the fresh femora were measured with an electronic caliper. After preservation, tibial bones were decalcified in 5% EDTA at room temperature. Paraffin-embedded coronal slices 10 μm thick from proximal tibia were taken and stained with hematoxylin/eosin and alcian blue for histological assessment of the epiphyseal plate. A similar procedure was repeated after 56 days in the second phase of the experiment.
Results: The femora of hydrocortisone-treated rats were significantly shorter than those of controls, but after concomitant administration of salmon calcitonin the difference was insignificant. The histological assessment showed that depressed function of growth plate in hydrocortisone-treated animals after both phases of the experiment was partially reversed by salmon calcitonin treatment.
Conclusions: Prolonged concomitant treatment with high-doses of salmon calcitonin partially reverses glucocorticoid-induced depression of rat growth plate. **(Pol J Endocrinol 2011; 62 (1): 18–23)**

Key words: *growth plate, glucocorticosteroids, calcitonin*

Streszczenie

Wstęp: Glukokortykosteroidy hamują wzrost kości, a podawanie kalcytoniny rosnącym szczurom zwiększa ich długość.
Celem pracy była ocena wpływu kalcytoniny na zahamowanie wzrastania szczurów poddanych działaniu hydrokortyzonu. Porównano długości kości udowych i obrazy histologiczne chrząstki wzrostowej bliższej przynasady piszczeli w badanych grupach. Doświadczenie przeprowadzono w dwóch fazach 28- i 56-dniowych.
Materiał i metody: Rosnące szczury szczepu Wistar w sposób zrandomizowany podzielono na dwie grupy kontrolne i cztery badane. Szczurom dwóch pierwszych grup badanych podawano hydrokortyzon *i.p.* dwa razy dziennie. Szczury pozostałych grup badanych poza hydrokortyzonem otrzymywały kalcytoninę łososiową *s.c.* dwa razy dziennie. Szczury z grup kontrolnych otrzymywały placebo. Pierwszą fazę eksperymentu zakończono po 28 dniach. Po pobraniu długość kości udowych zmierzono elektroniczną suwmiarką. Po utrwaleniu kości piszczelowe odwapniono w 5% EDTA. Preparaty wybarwiono za pomocą H+E i błękitu alcjanu. Procedurę powtórzono po 56 dniach jako drugą fazę doświadczenia.
Wyniki: Kości udowe zwierząt, którym podawano hydrokortyzon były istotnie statystycznie krótsze niż w grupie kontrolnej. Zależność ta nie była istotna w grupie zwierząt poddanych działaniu kalcytoniny. Ocena histologiczna preparatów chrząstki wzrostowej obu faz doświadczenia pokazała częściowe odwrócenie negatywnego wpływu hydrokortyzonu u zwierząt, którym podawano kalcytoninę.
Wnioski: Wysokie dawki kalcytoniny łososiowej zmniejszają zahamowanie czynności chrząstki wzrostowej szczurów wywołane przewlekłą glukokortykoterapią. **(Endokrynol Pol 2011; 62 (1): 18–23)**

Słowa kluczowe: *chrząstka wzrostowa, glukortykosteroidy, kalcytonina*

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Introduction

The epiphyseal (growth) plate is the highly organised and stratified growth organ located between the epiphysis and the metaphysis of the long bones in skeletally immature animals and humans. Longitudinal growth occurs within the growth plate due to orchestrated events, predominantly multiplication of specialised stem chondrocytes (germinative, cambial layer) that consecutively organise in columns, thus creating a so-called transitional layer of growth plate. More mature chondrocyte cells (hyperthrophic layer) that approach methaphyseal highly vascularised spongiosa undergo gradual apoptosis combined with calcification of the underlying matrix (degenerative layer) [1–3]. Subsequent invasion of proliferating capillaries carrying chondroclasts and osteoclasts in order to remove dead chondrocytes and calcified matrix together with cells of osteoblastic lineage occurs from the methaphysis [4]. Replacing the calcified chondrogenic scaffold, new trabeculae of spongiotic bone in continuity with the methaphyseal spongiosa are formed — a process called enchondral ossification maintaining longitudinal growth of bones occurs [5, 6].

This complicated physiological phenomenon is regulated by several factors of hormonal, local, environmental and nutritional origins [7–16]. One of the most well-known and clinically important factors that decrease longitudinal growth causing short stature are corticosteroids which could be of excess due to both endo- and exogenic origin [17]. Calcitonin, a polypeptide hormone produced under physiological conditions mainly by C cells of the thyroid gland, apart from its direct anti-osteoclastic action exerts anabolic effect on chondroblastic and osteoblastic lineage. Synthesized salmon calcitonin has been shown to be a potent agent used in several animal studies, exerting its important role in the maturation of the growth plate [18–21].

The aim of our study was to determine the effects on the growth plate of concomitant administration of salmon calcitonin in hydrocortisone-treated growing male rats.

Material and methods

Fifty six young adult male Wistar rats (body mass ca 300 g, aged 12 weeks) were randomly divided into two control groups called C1 and C2 (n = 8) and four experimental groups (n = 10). Rats in the first two experimental groups (H1 and H2) were treated i.p. b.i.d. with hydrocortisonum hemisuccinatum (Hydrocortisonum hemisuccinatum, Polfa, Poland) 10 mg/kg. Rats in the other experimental groups (H-C1 and H-C2), other than treatment with hydrocortisone as above, were also administered s.c. b.i.d. 15 UI/kg s.c., salmon calcitonin (Miacalcic, Sandoz, Basle, Switzerland). The controls received appropriate vehicula. The rats were kept under standardised conditions, food and water *ad libidum*, at room temperature with light 12 h/24 h.

The first phase of the experiment was completed after 28 days and right femora and tibiae were harvested from groups (C1, H1, H-C1) and preserved in 4% buffered formalin. Lengths of the fresh femora were measured with an electronic caliper. After preservation, tibial bones were decalcified in 5% EDTA at room temperature. Paraffin- embedded slices 10 μm thick from proximal tibia in order to assess growth plate were taken under standardised coronal sectioning and stained with hematoxylin/eosin and alcian blue for glycosaminoglycans. Standardised histological assessment of the epiphyseal plate pictures under different magnifications was then performed. A similar procedure was repeated after 56 days in the second phase of the experiment (in the groups C2, H2, H-C2). To measure differences in length of the femora, one-way Anova was used (Statistica 6.0). $P \leq 0.05$ was regarded as significant.

Results

The histological pictures revealed depressed function of growth plate in hydrocortisone-treated animals after both phases of the experiment. In hydrocortisone-calcitonin-treated rats, depression of growth plate cellular activity was less marked. The femora of hydrocortisone-treated rats were significantly shorter than those of controls, but after concomitant administration of salmon calcitonin, the difference proved to be insignificant (Figs. 1–6, Table I).

Discussion

Several clinical conditions such as juvenile rheumatoid arthritis, asthma, renal and other organ transplantations require prolonged glucocorticosteroid therapy. Under such circumstances, retardation of growth occurs [17]. Glucocorticosteroid receptors have been demonstrated in the proliferating and hypertrophic zones of rat and human growth plates [22] and it has been shown that they are capable of biphasically modifying expression of growth hormone receptor, thus directly affecting activity of the growth plate [23]. Joux et al. proved the influence of dexamethasone treatment on suppression of local IGF-I production and expression of growth hormone and IGF-I-receptor in cultured rat chondrocytes [24]. This view was supported by Olney who claimed that at the local level glucocorticosteroids decreased IGF-I production, induced IGF-I resistence and reduced the production of C-type natriuretic peptide, which resulted in a reduction of chondrocyte prolifera-

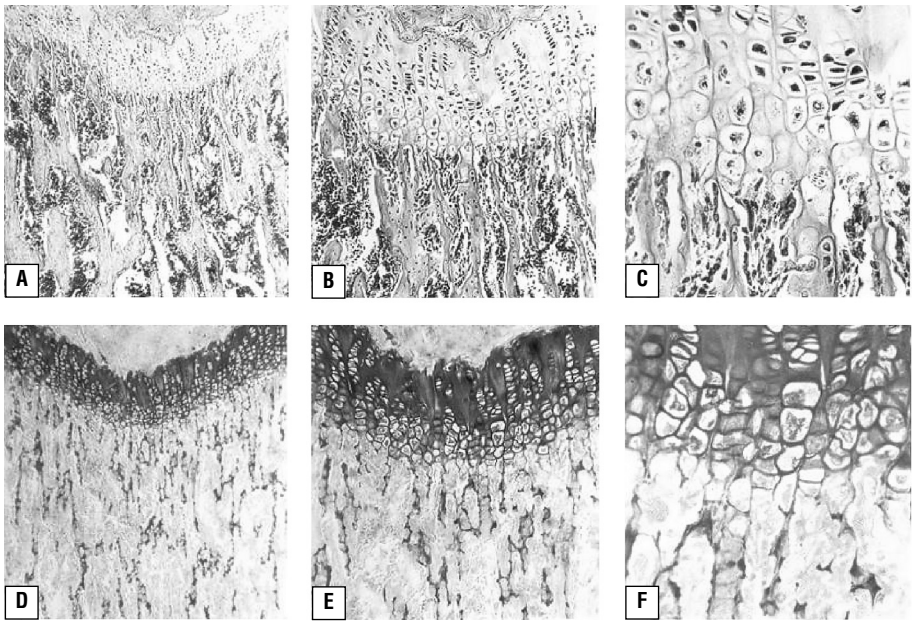

Figure 1. *Control group 1 (C1). Histological pictures of epiphyseal plate and trabecular bone of proximal tibiae after first phase of experiment (four weeks). Specimens stained with H+E (**A, B, C**) and alcian blue (**D, E, F**). After staining with H+E (A — 50 ×, B — 100 ×, C — 250 ×) normal layers of cartilage and trabeculae are clearly visible. Alcian blue staining revealed cartilage abundant in acid mucopolysaccharides (D — 50 ×, E — 100 ×, F — 250 ×)*

Rycina 1. *Obrazy histologiczne chrząstki wzrostowej i kości gąbczastej bliższego końca piszczeli szczura przekroju w płaszczyźnie czołowej po pierwszej fazie doświadczenia (4 tygodnie) w grupie kontrolnej (C1). Obrazy prawidłowe. Zachowaną warstwową budowę chrząstki wzrostowej oraz sąsiadujące z nią regularnie ułożone beleczki kostne wyraźniej przedstawiają kolejne powiększenia (H + E, A, B, C, pow. 50, 100 i 250 ×). Barwienie błękitem alyjanu ujawnia mukopolisacharydy kwaśne szczególnie obfite w chrząstce wzrostowej (D, E, F, pow. 50, 100 i 250 ×)*

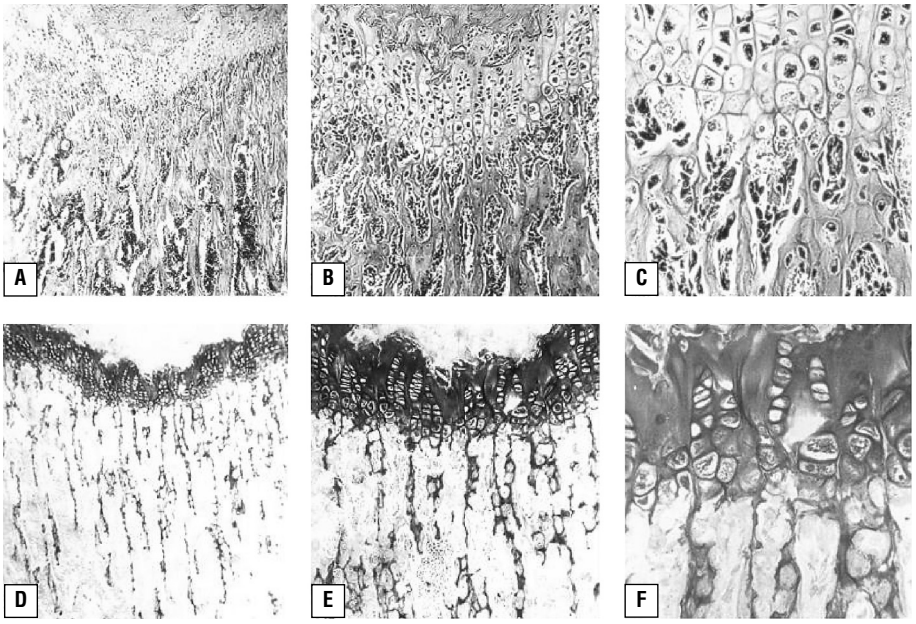

Figure 2. *Group H1. After four weeks of corticosteroidotherapy, the growth plate has become thinner with flattened cells, particularly in the germinative layer. (H + E; A — 50 ×, B — 100 ×, C — 250 ×). Alcian blue staining revealed growth cartilage thinner than in the control group and unresorbed calcified cartilage close to methaphyseal trabeculae (D — 50 ×, E — 100 ×, F — 250 ×)*

Rycina 2. *Obrazy histologiczne chrząstki wzrostowej i kości gąbczastej bliższego końca piszczeli szczura po pierwszej fazie doświadczenia (4 tygodnie) po podawaniu hydrokortyzonu przez 4 tygodnie (grupa H1). Osteoblasty i osteocyty są mniej liczne, a ich jądra częściej przybierają kształt owalny. Chrząstka wzrostowa cieńsza ze spłaszczonymi komórkami, zwłaszcza w warstwie rozrodczej (H + E, A, B, C, pow. 50, 100 i 250 ×). Barwienie błękitem alcyjanu podkreśla mniejszą, w porównaniu z kontrolą, grubość warstwy chrząstki wzrostowej oraz ujawnia większe nagromadzenie niezresorbowanej uprzednio zwapniałej chrząstki w bezpośrednim sąsiedztwie beleczek kostnych części przynasadowej (D, E, F, pow. 50, 100 i 250 ×)*

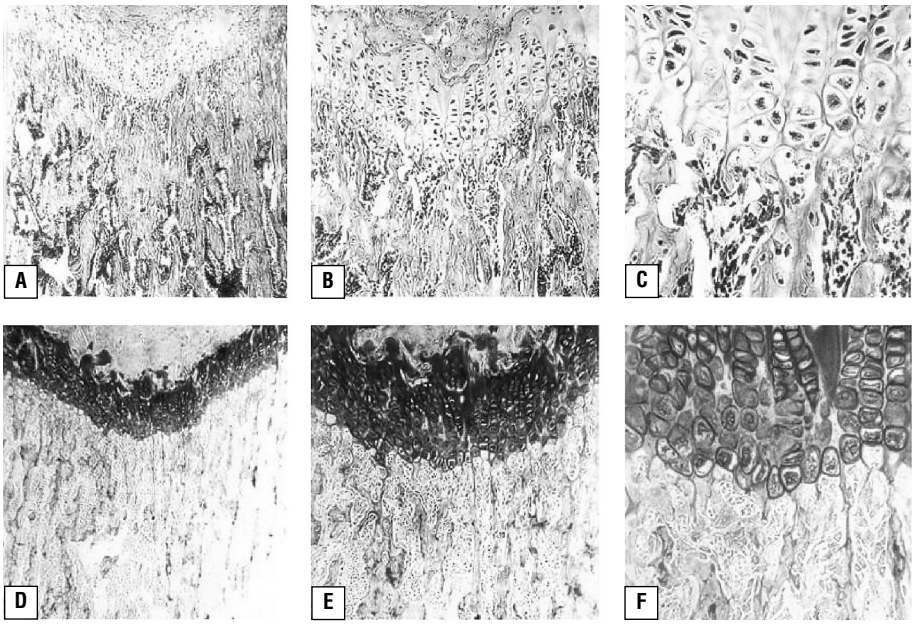

Figure 3. *Group H-C1. After four weeks of concomitant treatment with calcitonin, the effect of glucocorticosteroids was partially reversed. Chondrocytes were frequent, with normal shape and nuclei strongly stained with hematoxylin. Thickened trabeculae were frequently connected. Double chondrons and chondron groups were quite frequent (A — 50 ×, B — 100 ×, C — 250 ×). Alcian blue staining revealed a similar picture. Growth plate had normal thickness with more cells than in group H1. Almost no calcified matrix fragments were visible (D — 50 ×, E — 100 ×, F — 250 ×).*

Rycina 3. *Po 4 tygodniach stosowania hydrokortyzonu i syntetycznej kalcytoniny łososiowej (grupa H-C1) komórki chrzęstne są liczne, prawidłowego kształtu, a ich jądra dobrze barwią się hematoksyliną. Liczne są chondrony dwukomórkowe i skupiska komórek chrzęstnych. Pogrubiałe beleczki kostne często łączą się ze sobą (H + E, A, B, C, pow. 50, 100 i 250 ×). Barwienie błękitem alyjanu ujawnia prawidłową grubość chrząstki wzrostowej oraz zwiększenie liczby jej komórek w odniesieniu do grupy hydrokortyzonowej. W sąsiedztwie głębiej położonych beleczek niemal nie występują fragmenty odpowiadające zwapniałej macierzy chrzęstnej (D, E, F, pow. 50, 100 i 250 ×)*

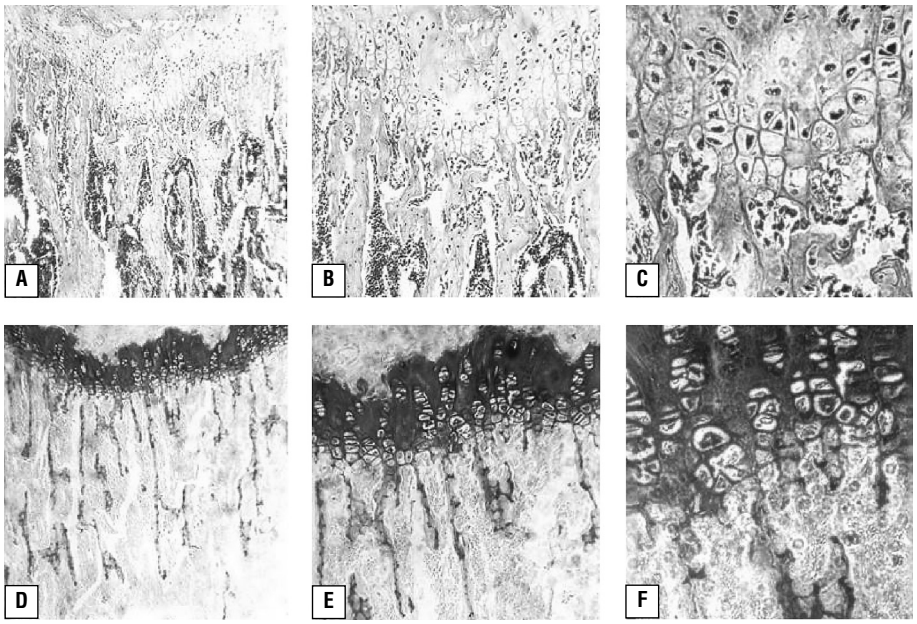

Figure 4. *Control group 2 (C2). After eight weeks, trabeculae were thicker but less frequent than in control group C1 (A — 50 ×, B — 100 ×, C — 250 ×). Alcian blue staining revealed abundant acid mucopolysaccharides in cartilage and in the layer of calcified matrix. Growth plate appeared to be slightly thinner than in C1 (D — 50 ×, E — 100 ×, F — 250 ×)*

Rycina 4. *Obraz chrząstki wzrostowej oraz kości beleczkowej bliższej części piszczeli szczurów grupy kontrolnej po II fazie doświadczenia (grupa C2). Beleczki kostne są grubsze, lecz mniej liczne niż na obrazach preparatów z grupy C1 (ryc. 1). Ich osteoblasty i osteocyty są okrągłe, a substancja jąder komórkowych barwi się intensywnie hematoksyliną (H + E, A, B, C, pow. 50, 100 i 250 ×). Barwienie błękitem alcyjanu ujawnia mukopolisacharydy kwaśne, szczególnie obfite w tkance chrzęstnej, także w jej strefie odpowiadającej zwapniałej macierzy (D, E, F, pow. 50, 100, 250 ×)*

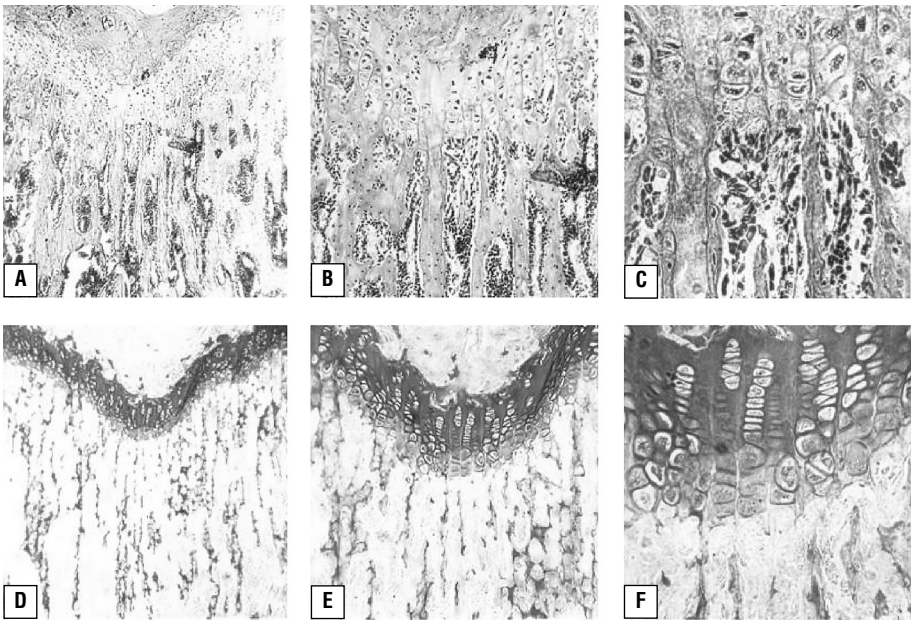

Figure 5. *Group H2. After eight weeks of corticosteroidotherapy, the growth plate appeared thinner with flattened and less frequent cells, particularly in the germinative layer. (H + E; A — 50 ×, B — 100 ×, C — 250 ×). Alcian blue staining revealed a similar picture. Growth cartilage layer was thinner than in control group with flattened cells and unresorbed calcified cartilage close to methaphyseal trabeculae (D — 50 ×, E — 100 ×, F — 250 ×)*

Rycina 5. *Grupa H2. Po 8 tygodniach stosowania hydrokortyzonu w odniesieniu do obrazów kontrolnych zwraca uwagę ścieńczenie chrząstki wzrostowej oraz spłaszczenie jej mniej licznych komórek, zwłaszcza warstwy rozrodczej. Osteoblasty i osteocyty są mniej liczne a ich jądra częściej przybierają kształt owalny lub nieregularny (H + E, A, B, C, pow. 50, 100 i 250 ×). Barwienie błękitem alcjanu podkreśla mniejszą w porównaniu z kontrolą grubość warstwy chrząstki wzrostowej, spłaszczenie jej komórek oraz ujawnia większe nagromadzenie tkanki odpowiadającej niezresorbowanej zwapniałej jej części w bezpośrednim sąsiedztwie beleczek kostnych części przynasadowej (D, E, F, pow. 50, 100, 250 ×)*

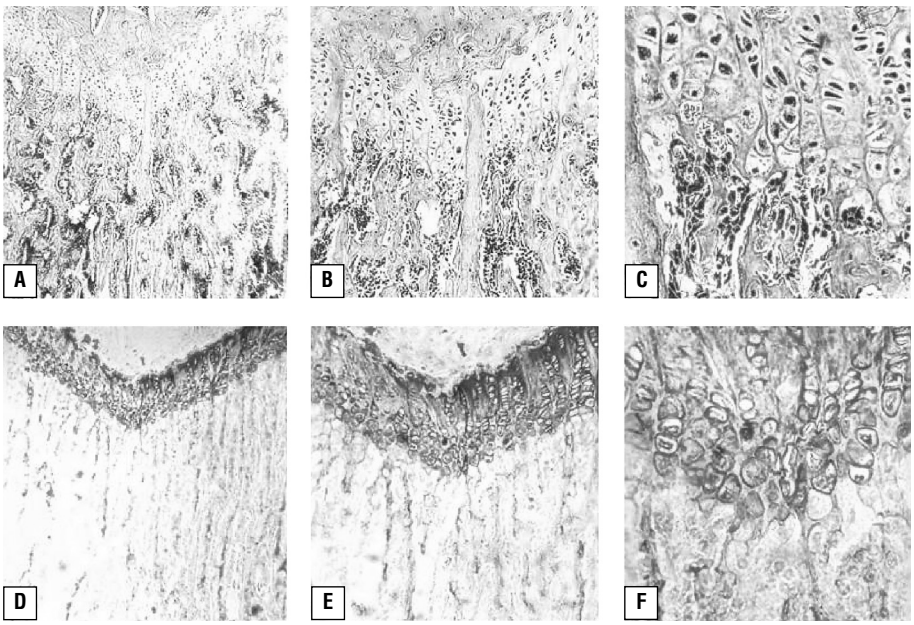

Figure 6. *Group H-C2. After eight weeks of concomitant treatment with calcitonin, histological pictures were similar to group H-C1. Steroid effect was partially reversed. Chondrocytes were frequent and normally shaped with nucleus strongly stained with hematoxylin. Thick trabeculae of young bone were often connected (A — 50 ×, B — 100 ×, C — 250 ×). Alcian blue staining revealed apparently normal thickness of growth plate with less staining affinity, probably due to less intensive growth of older animal. Chondral cells were more frequent than in H2. Almost no calcified matrix fragments were observed (D — 50 ×, E — 100 ×, F — 250 ×)*

Rycina 6. *Grupa H-C2. Po 8 tygodniach stosowania hydrokortyzonu i syntetycznej kalcytoniny łososiowej obrazy mikroskopowe chrząstek wzrostowych bliższej części kości piszczelowych nie różnią się istotnie od obserwowanych w grupie H-C1 (ryc. 4). Komórki chrzęstne są liczne, prawidłowego kształtu, a ich jądra dobrze barwią się hematoksyliną. Grube beleczki młodej kości często łączą się ze sobą (H + E, A, B, C, pow. 50, 100 i 250 ×). Barwienie błękitem alyjanu ujawnia prawidłową grubość chrząstki wzrostowej i jej nieco mniejsze powinowactwo do barwnika niż w grupie H-C1, wynikające z mniej intensywnego wzrastania starszych zwierząt II fazy doświadczenia. Komórki chrzęstne w porównaniu z grupą H-2 są liczniejsze. W sąsiedztwie głębiej położonych beleczek niemal nie występują fragmenty odpowiadające zwapniałej macierzy chrzęstnej (D, E, F, pow. 50, 100 i 250 ×)*

Table I. *Mean lengths of measured femora (mm, SD in brackets, *p ≤ 0.05)*

Tabela I. *Średnie długości zmierzonych kości udowych (mm, SD w nawiasach, *p ≤ 0,05)*

Controls (n = 8)	Hydrocortisone-treated (n = 10)	Hydrocortisone and calcitonin-treated (n = 10)
	After four weeks	
36.75 (0.81)	35.59* (0.71)	35.97 (1.23)
	After eight weeks	
38.63 (0.63)	37.40* (0.99)	37.74 (0.73)

tion, matrix synthesis and hypertrophy [25]. Sivestrini et al. demonstrated that high doses of cortisone reduced growth plate width, resulting in growth retardation [22].

Our studies fully supported these findings and (similarly to the observations of Annefeld) indicated that the most likely explanation of this phenomenon was reduced chondrocyte proliferation and increased apoptosis in terminal hypertrophic chondrocytes [26]. That phenomenon was observed also by Chrysis et al. [27], whose study showed that dexamethasone induced apoptosis in proliferative chondrocytes through activation of caspases and suppression of the akt-phosphatidylinositol 3′-kinase signalling pathway.

The study by Khalli et al. [18] demonstrated that a high dose (6 IU per rat) of salmon calcitonin administered for 12, 18 and 24 weeks enhanced the number of 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.

The results of our study support a beneficial effect of salmon calcitonin on longitudinal skeletal growth in long-term hydrocortisone-treated young adult male rats. Both histological pictures of the epiphyseal plate and results of measurements of length of the femora supported the thesis that high doses of salmon calcitonin partially reversed growth inhibition caused by hydrocortisone both after 28 and 56 days of treatment.

Although the usefulness of these observations as regards humans requires separate study, these results seem to be of some clinical importance as glucocorticosteroids are often used in the treatment of chronic inflammatory conditions in children.

References

1. Hunziker EB. Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc Res Tech 1994; 28: 505–519.
2. Kamber NF. Cell kinetics and the control of bone growth. Acta Paediatr 1993; 82 Suppl 391: 61–65.
3. Horton Jr WE, Feng L, Adams C. Chondrocyte apoptosis in development, aging and disease. Matrix Biol 1998; 17: 107–115.
4. Kember NF, Sissons HA. Quantitative histology of the human growth plate. J Bone Joint Surg Br 1976; 58-B: 426–435.
5. Lewinson D, Silbermann M. Chondroclasts and endothelial cells collaborate in the process of cartilage resoption. Anat Rec 1992; 233: 504–514.
6. Vu TH, Shipley JM, Bergers G et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 1998; 93: 411–422.
7. Abad V, Meyers JL, Weise M et al. The role of the resting zone in growth plate chondrogenesis. Endocrinology 2002; 143: 1851–1857.
8. Gafni RI, Weise M, Robrecht DT. Catch-up growth is associated with delayed senescence of the growth plate in rabbits. Pediatr Res 2001; 50: 618–623.
9. Minina E, Kreschel C, Naski MC et al. Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. Dev Cell 2002; 3: 439–449.
10. Wang W, Xu J, Kirsch T. Annexin-mediated Ca2_influx regulates growth plate chondrocyte maturation and apoptosis. J Biol Chem 2003; 278: 3762–3769.
11. Boyan BD, Sylvia VL, Dean DD et al. 24,5(OH)(2)D(3) regulates cartilage and bone via autocrine and endocrine mechanism. Steroids 2001; 66: 363–374.
12. Cancedda R, Descalzi Cancedda F, Castagnola P. Chondrocyte differentiation. Int Rev Cytol 1995; 159: 265–358.
13. Hering TM. Regulation of chondrocyte gene expression. Front Biosci 1999; 4: D743–D761.
14. Stevens DA, Williams GR. Hormone regulation of chondrocyte differentiation and endochondral bone formation. Mol Cell Endocrinol 1999; 151: 195–204.
15. Robson H, Siebler T, Shalet SM et al. Interactions between GH, IGF-I, glucocorticoids, and thyroid hormones during skeletal growth. Pediatr Res 2002; 52: 137–147.
16. Kronenberg HM. Developmental regulation of the growth plate. Nature 2003; 423: 332–336.
17. Bello CE, Garret SD. Therapeutic issues in oral glucocorticoid use. Lippincotts Prim Care Pract 1999; 3: 333–341.
18. Khaldi L, Karachalios, Galanos A et al. Morphometic changes in the epiphyseal plate of the growing and young adult male rat after long-term salmon calcitonin administration. Calcif Tissue Int 2005; 76: 426–432.
19. Wallach S, Rousseau G, Martin L et al. Effects of calcitonin on animal and in vitro models of skeletal metabolism. Bone 1999: 25: 509–516.
20. Ishikawa Y, Wu LN, Genge BR et al. Effects of calcitonin and parathyroid hormone on calcification of primary cultures of chicken growth plate chondrocytes. J Bone Miner Res 1997; 12: 356–366.
21. Naot D, Cornish J. The role of peptides and receptors of the calcitonin family in the regulation of bone metabolism Bone 2008; 43: 813–818.
22. Silvestini G, Ballanti P, Patacchioli FR et al. Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. Bone 2000; 26: 33–42.
23. Vottero A, Kimchi-Safarty C, Kratzsch J et al. Transcriptional and translational regulation of the splicing isoforms of the growth hormone receptor by glucocorticoids. Horm Metab Res 2003; 35: 7–12.
24. Jux C, Leiber K, Hugel U et al. Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. Endocrinology 1998; 139: 3296–3305.
25. Olney RC. Mechanism of impaired growth: effect of steroids on bone and cartilage Horm Res 2009; 72: 30–35.
26. Annefeld M. Changes in rat epiphyseal cartilage after treatment with dexamethasone and glycosaminoglycan-peptide complex. Pathol Res Pract 1992; 188: 649–652.
27. Chrysis D, Zaman F, Chagin AS et al. Dexamethasone induces apoptosis in proliferative chondrocytes trough activation of caspases and suppression of the akt-phosphatidylinositol 3′-kinase signaling pathway. Endocrinology 2005; 146: 1391–1397.