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Intermittent Administration of the Circulating Form of Human Parathyroid Hormone (hPTH-1-37) Prevents Bone Loss in Ovariectomized Rats

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Abstract
The circulating hormonal form of human parathyroid hormone (hPTH-1-37) has been assessed *in vitro* as well as *in vivo* in the ovariectomized rat, a model for post-menopausal osteoporosis. *In vitro*, hPTH-1-37 induces a dose-dependent cAMP formation and increases vitality as well as alkaline phosphatase activity in UMR106 osteosarcoma cells. Differentiation and proliferation of osteoclasts in rat bone marrow-derived stem cell preparations are decreased. Daily hPTH-1-37 s.c. administration in ovariectomized rats for 60 days results in augmented formation of new bone, in amplified femural bone density, and in thickening of the calvaria.

Key words: Human Parathyroid Hormone (hPTH-1-37), Bone Loss

Introduction

In mammals, parathyroid hormone (PTH) regulates calcium homeostasis. PTH is synthesized in the parathyroid gland as a precursor molecule, prepro-PTH [1]. After cleavage of the prepro-sequence, PTH is stored in dense-core secretory vesicles. Depending on the serum calcium level, these vesicles fuse with the plasma membrane and PTH is secreted.

The native, circulating peptide of PTH exists in several molecular forms, the largest consisting of 84 amino acids. However, N-terminal PTH fragments are responsible for its metabolic effects [2]. Under hypocalcemic conditions, PTH is capable of initiating the release of calcium from bone. Simultaneously, it upregulates the synthesis of α-1,25-dihydroxy-vitamin D_3 ($VitD_3$) in the kidney. This leads to enhanced enteral calcium absorption and decreased renal excretion of calcium whereas phosphate excretion is diminished. Hence, physiological calcium levels, are rapidly restored [3].

The ostensibly paradox osteoanabolic effect of parathyroid hormone is long known. Since then, substantial research has been performed and now, PTH is a promising candidate for the treatment of osteoporosis [4]. It has been demonstrated that intermittent injections of PTH elicit an osteoanabolic effect in cancellous bone whereas continuous administration of PTH by infusion produces catabolic effects in particular at the cortical bone [5]. Different studies showed an anabolic effect at the corticalis after intermittent administration of PTH [6, 5, 7]. It has been shown that PTH causes a more pronounced osteoanabolic effect than estrogen or bisphosphonates with such treatment strategies [8]. The combination of PTH and estrogen results in an additive osteoanabolic effect [9]. Furthermore, Kimmel et al. report that synthetic hPTH-1-34 is more potent than recombinant hPTH-1-84 [10] suggesting differential pharmacodynamic characteristics of various N-terminal PTH fragments.

The biologically active circulating form hPTH-1-37 was isolated from human hemofiltrate [11], therefore being the putative native hormonal peptide form. However, osteoanabolic effects of hPTH-1-37 in the ovariectomized rat model have not yet been described. In this study, we analyzed the effect of hPTH-1-37 on bone density and turnover as well as pharmacodynamic properties of different length N-terminal PTH fragments *in vitro*.

Materials and Methods

Drugs and Reagents

For *in vitro* experiments, PTH-1-37 and PTH-1-34 were used. Both fragments were produced by standard Fmoc synthesis at IPF pharmaceuticals, Hannover, Germany. All other drugs and chemicals were from Th. Geyer, Hamburg, Germany. The cAMP ELISA-Kit was from IHF GmbH, Hamburg, Germany.

Cell Culture

UMR-106 rat osteosarcoma cells (which express the PTH type I receptor) were cultivated in α-MEM without phenol red (Gibco BRL) containing 10% FCS, penicillin, streptomycin and glutamine. Cells were washed in PBS twice and incubated with different concentrations of hPTH-1-34 or hPTH-1-37 in α-MEM without phenol red for 30 min or α-MEM alone. Supernatants were removed and either used di-

rectly for determination of cAMP or frozen at -20 °C until use.

Bone marrow stem cells were also cultured in α-MEM without phenol red (Invitrogen, Karlsruhe, Germany), 10% FCS (BioChrom, Berlin, Germany; inactivated for 45 min at 56 °C) with penicillin, streptomycin, glutamine, amphotericin B (fungizone) [α-MEM(-)] and ascorbic acid (50 µg/ml) [α-MEM(+)]. These bone marrow cells were obtained from tibia and femur of adult rats. Bone was dissected free of soft tissue. The distal end was opened and marrow was flushed out with 2 ml α-MEM(+). A single cell suspension from 4 bones was made by repeated flushing through a pipette and resuspended in an end volume of 25 ml. 500 µl per well from this cell suspension (approx. 5 mio cells/ml) were applied to a single well of a 48-well plate and cultured overnight. Then the plates were washed once with 300 µl α-MEM(-), the wells were filled with 500 µl α-MEM(+) and the test substances were added. After 3 days, the medium was changed and the cells were cultured for a further 4 days in the presence of test substances. At the end of the experiment, viability, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activity were determined.

Viability, Proliferation

After washing with 200 µl/well α-MEM(-), WST-1 (Roche Diagnostics, Penzberg, Germany) diluted 1:10 in α-MEM(-) was applied. Then the cells were incubated for 30 - 60 min at 37 °C and the extinction was measured using an ELISA reader at 450/630 nm.

ALP Activity

Cells were fixed in ethanol (95%) for 10 min, equilibrated for 5 min with 200 µl per well carbonate-bicarbonate buffer (SIGMA, Deisenhofen, Germany) C-3041 and incubated 30 - 60 min with substrate (paranitrophenylphosphate; 1 mg/ml) in carbonate-bicarbonate buffer. Absorption was measured at 405 nm.

TRAP Staining

After the ALP reaction, the cells were fixed with 0.5% Triton in PBS for 10 min, washed once with distilled water and stained with a tartrate-resistant acid phosphatase kit according to the supplier's protocol (SIGMA cat. No. 387-A, Deisenhofen, Germany). The staining solution contained tartrate. After incubation for 1 h at 37 °C the wells were washed with distilled water, stained with Mayer's hematoxylin for 30 min in the dark and washed twice with tap water. All TRAP-positive cells with two or more nuclei in each well were counted and defined as osteoclasts.

cAMP-ELISA

The cAMP-ELISA was carried out essentially as described [12] but supernatants were used instead of cell extracts. Briefly, cells were treated with increasing doses of hPTH-1-34 and hPTH-1-37 for 30 min, 100 µl supernatant was acetylated and a 50 µl aliquot was pipetted onto a coated ELISA plate according to the supplier's protocol.

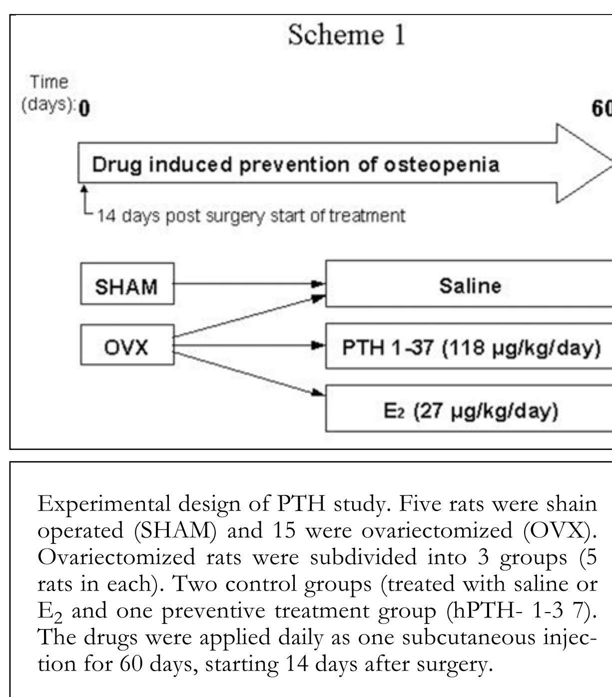
Animals

Twenty female, 12-week-old Wistar rats [five sham-operated (Sham) and 15 ovariectomized (OVX)] were obtained from Harlan-Winkelmann GmbH (Borchen, Germany). The rats were acclimatized for a period of 12 days before start of the study then divided into groups of 5 animals and kept in Macrolon® cages type IV (Ebeco Becker & Co GmbH, Castrop-Rauxel, Germany) with a 12 h light/12 h darkness cycle at 20 °C ± 3°C and relative humidity of 50% ± 10%. Food was standard rat food pellets (ssniff R+M Haltungsfutter, ssniff Spezialdiäten GmbH, Soest, Germany) and tap water, both offered *ad libitum*. Body weights were determined at the beginning of the study and weekly thereafter, always in the morning. Prior to its start, the study had been approved by the local animal protection committee.

Experimental Design

Drugs were applied daily as subcutaneous injection for 60 days, starting 14 days after surgery (Scheme 1). Treatment groups were Sham + NaCl (physiological saline), OVX + NaCl, OVX + 27 mg/kg/day 17-β-estradiol (E_2, Sigma-Aldrich, Taufkirchen, Germany) and OVX + 118 µg/kg/day synthetic hPTH-1-37 (IPF PharmaCeuticals GmbH, Hannover, Germany). Physiological saline was used as vehicle for hPTH-1-37 and E_2. Blood samples were collected retroorbitally on days 1, 7, 14, 21, 35 and 49. Serum was stored at -20 °C until analysis (total calcium and osteocalcin).

For the purpose of fluorescent labeling, rats received 7 mg tetracycline and 7 mg calcein (Sigma-Aldrich, Taufkirchen, Germany) intraperitoneally, dis-



Experimental design of PTH study. Five rats were shain operated (SHAM) and 15 were ovariectomized (OVX). Ovariectomized rats were subdivided into 3 groups (5 rats in each). Two control groups (treated with saline or E_2 and one preventive treatment group (hPTH- 1-3 7). The drugs were applied daily as one subcutaneous injection for 60 days, starting 14 days after surgery.

solved in physiological saline on days 49 and 56 of the experiment. The animals were killed on day 63 using ether anesthesia followed by decapitation. Femora and calvaria were dissected and freed from soft tissue. They were fixed separately in 10 ml phosphate-buffered paraformaldehyde (fixation solution changed every 3 days) and stored at 4 °C for 4 weeks. Finally, they were cleaned from remaining soft tissue and dried for determination of femur density, calvarial thickness, and X-ray analysis.

Measurement of Femur Density

Femur density was determined by the Mohr-scale principle ($r_{20°}$ = m/V). Dissected and fixed femora were dried at 120 °C for 4 h. The dry weight was measured and the volume was obtained by the Archimedes principle. Femur density (g/ml) is determined as bone dry weight (g) divided by bone volume (ml).

Radiological Analysis

X-ray analysis was performed using Philips equipment (Bucky Diagnostic, Miami, USA), Kodak cassette (X-Omatic Cassette, KP 68374-A, Vienna, Austria) and Kodak diagnostic film (InSight™ Imaging Film, Vienna, Austria). The parameters of the program were set to 44 kV; 0,63 mAs and 3,58 ms and the film focus distance was 35 cm.

Histology

Thin sections were prepared according to Kierdorf [13]. Fixed and dried femur was cut perpendicularly in the middle of the diaphysis. A slice of 2-3 mm was cut off and embedded in UHU®plus glue (UHU 2-Komponenten-Epoxy-Harz-Klebstoff, Bühl, Germany). After 24 h solidifying at room temperature, the object was ground using wet sandpaper in descending grit size (P240, 400, 600, 1000). Finally, a polished thin section was embedded in Entellan®neu (Merck, Darmstadt, Germany).

The calvaria were split along the mid-suture. One 3 mm wide strip was cut off and embedded using UHU®acrylit glue (UHU 2-Komponenten-Klebstoff, Bühl, Germany) and cured at room temperature for 24 h. In a similar way to the femur thin sections, the object was ground with wet sandpaper in descending grit size (P180, 240, 400, 600, 1000).

Histomorphometrics of the Femur and Measurement of Calvaria Thickness

The distance between fluorescent labels represents the amount of bone which is deposited in the time interval between tetracycline and calcein administration. This space was determined using thin sections of femur diaphysis, a fluorescence Axiophot microscope, Plan Neofluar objective (63x/1,2) and E-PI 10x/20 measuring ocular (Zeiss, Oberkochen, Germany). For every thin section, the distance was measured in 6 different areas and values were averaged.

Calvaria thickness was measured using thin sections, in the Zeiss Axiophot light microscope, with an Achroplan objective (10x/0,25 PH1) and an E-PI 10x/20 measuring ocular. The measuring zone was bordered by the coronal (2 mm rostral) and the parietal (2 mm caudal) suture. Three reading points with the same distance were determined and values were averaged.

Measurement of Serum Osteocalcin

Serum osteocalcin was determined using a commercially available enzyme-linked immunosorbent assay (Rat-MID™ Osteocalcin ELISA, Osteometer Bio-Tech, Herlev, Denmark). The concentration of osteocalcin was expressed as ng equivalents of rat osteocalcin per ml.

Statistical Analysis

Results are presented as means ± S.E.M. Effects of treatment were analyzed by one-way analysis of variance (ANOVA), $p \leq 0.05$ is considered statistically significant. Whenever statistically significant differences were observed between the various experimental groups by ANOVA, individual differences were assessed by the Dunnett Test (using GraphPad Prism 3.0).

Results

In vitro Effects of hPTH

Both, hPTH-1-34 and hPTH-1-37 dose-dependently induced elevation of the second messenger cAMP in UMR-106 rat osteosarcoma cells, a well-established cell model to test PTH mediated cAMP production (Fig. 1) [14]. The EC50 values for cAMP-production were 19 and 33 nM, respectively ($p \geq 0.05$ in ANOVA).

We compared the influence of hPTH-1-34 and hPTH-1-37 on the number of osteoclasts formed (bone catabolic cells derived from hematopoietic precursor cells). Interestingly, hPTH-1-37 more efficiently

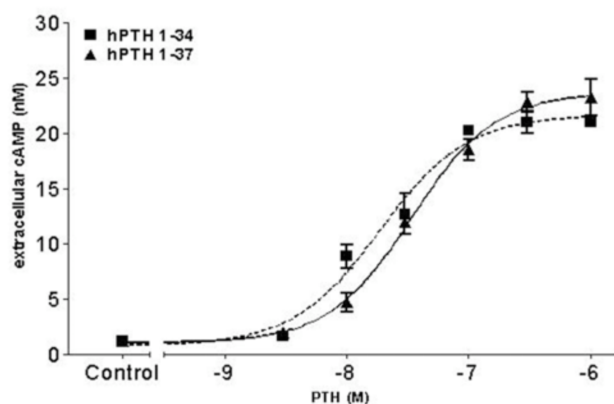


Fig. 1. cAMP levels after application of hPTH-1-34 or hPTH-1-37 onto UMR-106 cells. Both agents induce a dose-dependent increase of cAMP levels. The figure shows one of three independent experiments (means + SD; N = 3). The curves and the EC50 values are significantly different.

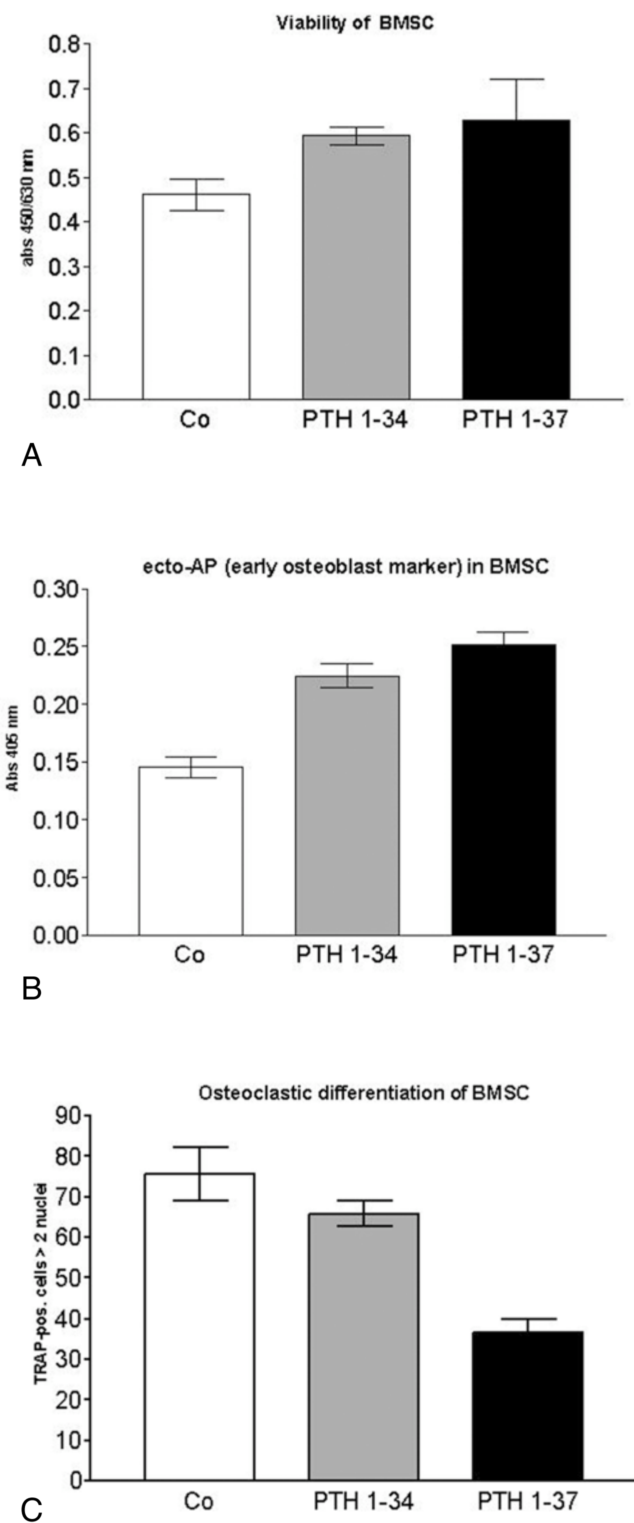


Fig. 2. A) Cell viability as measured by WST-1 activity. Both hPTH-1-34 ($p < 0.05$) and hPTH-1-37 ($p < 0.01$) induce an increase in WST-1 activity but do not sigificantly differ from each other. The figure shows pooled data from three independent experiments (means + SD; N = 9). B) Alkaline phosphatase activity. Both hPTH-1-34 and hPTH-1-37 induce an increase in AP activity. The figure shows pooled data from three independent experiments (means + SD; N = 9). C) hPTH-1-37 reduces the number of osteoclasts in BMST cultures in comparison to control and hPTH-1-34-treated cultures. The figure shows pooled data from three independent experiments (means + SD; N = 9).

reduced the number of osteoclasts than hPTH-1-34. The observed difference in inhibition of osteoclastic differentiation by 50% between the two compounds was statistically significant at $p \leq 0.01$ (Fig. 2c).Complementary to these findings, hPTH-1-37 increased ecto-alkaline phosphatase activity (Fig. 2b), an early osteoblast marker, to a slightly larger extent than hPTH-1-34 by 45%. However, this difference failed to reach conventional levels of statistical significance ($p \geq 0.05$).

Both PTH forms induced an increased viability of bone marrow derived stem cells ($p \leq 0.05$ and $p \leq 0.01$ compared to controls) but there was no clear differential effect between the two PTH forms (Fig. 2a).

Based on these *in vitro* effects, we assessed hPTH-1-37 in the ovariectomized rat, an animal model for postmenopausal osteoporosis.

Femur Density and Radiological Analysis

Ovariectomy caused a non-significant decrease in femur density (Sham NaCl, 1.51 ± 0.05 g/ml; OVX NaCl, 1.47 ± 0.05 g/ml) over the limited observation period. However, hPTH-1-37 treatment led to a significant increase in femur density (OVX PTH, 1.67 ± 0.04 g/ml; OVX NaCl versus OVX PTH, $p < 0.01$). E_2 did not increase femur density of ovariectomized rats significantly (OVX E_2, 1.43 ± 0.04 g/ml) (Fig. 3). These results are supported by X-ray analyses. The OVX NaCl and OVX E_2 groups show a clear reduction of cortical thickness and bone density of the popliteal surface. In contrast, administration of

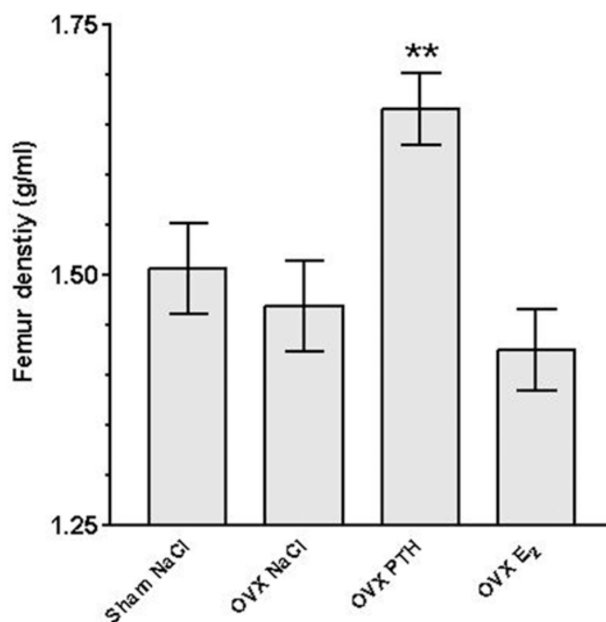


Fig. 3. Effects of sham operation, ovariectomy, ovariectomy + hPTH-1-37 treatment and ovariectomy + E_2 treatment on femur density. Results are shown as means ±-SEM (hPTH-1-37) N = 4 rats; all other groups N = 5 rats). Sham-operated rats treated with saline (NaCl) are compared to ovariectomized rats treated with saline. The remaining ovariectomized groups were compared to OVX control group (NaCl) (** P<0.01 versus OVX NaCl; ANOVA).

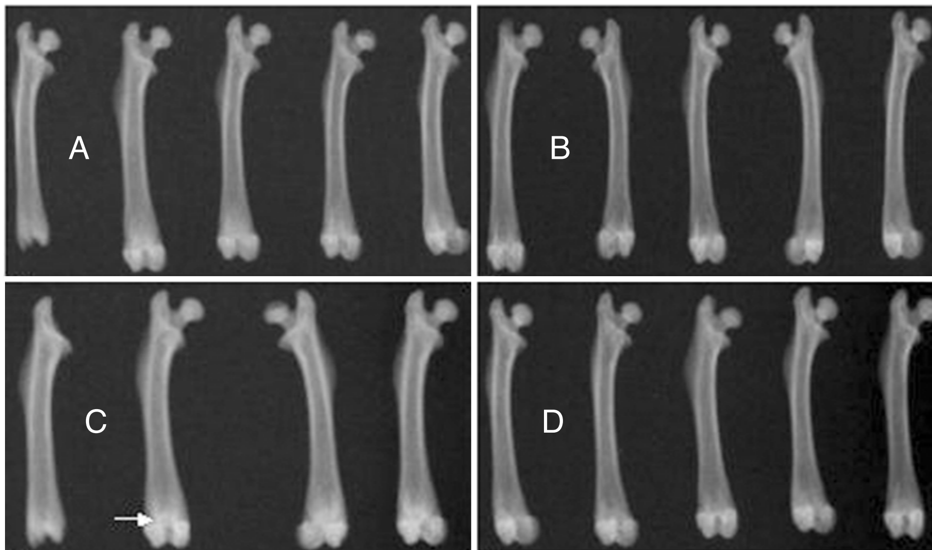


Fig. 4. X-ray analysis of femora from sham-operated (A), ovariectomized (B), ovariectomized + hPTH-1-37- treated (C) and ovariectomized + E_2-treated (D) rats.

hPTH-1-37 resulted in a distinct elevation of bone density at the popliteal surface and an increase in cortical thickness (Fig. 4; B - D).

Histomorphometry of the Femur and Thickness of the Calvaria

After ovariectomy, the distance between tetracycline and calcein fluorescent labels did not decline significantly (Sham NaCl, 11.68 ± 1.8 µm; OVX NaCl, 9.86 ± 2 µm). E_2 treatment causes no alterations (OVX E_2, 9.93 ± 1.3 µm), but hPTH-1-37 significantly increases the space between tetracycline and calcein fluorescent labels as markers for bone turnover of ovariectomized rats (OVX PTH, 12.85 ± 1.8 µm; OVX NaCl versus OVX PTH, $p < 0.05$) (Fig. 5).

Calvarial thickness was measured after fixation using thin sections. Ovariectomy significantly increased skull thickness (Fig. 8; Sham NaCl, 395 ± 59 µm; OVX NaCl, 471 ± 19.5 µm; Sham NaCl versus OVX NaCl, $p < 0.05$). E_2 treatment resulted in minor changes (OVX E_2 451 ± 23.5 µm versus OVX NaCl, 471 ± 19.5 µm) whereas hPTH-1-37 caused a significant increase in calvarial thickness (OVX PTH, 549 ± 36.4 µm; OVX NaCl versus OVX PTH, $p < 0.01$ (Fig. 6).

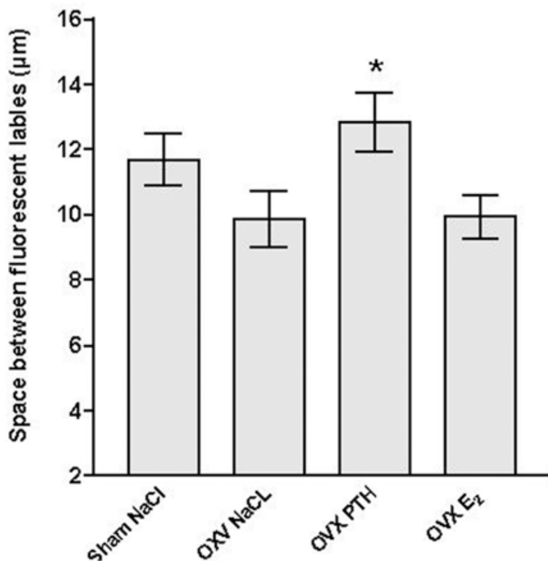


Fig. 5. Effects of sham operation, ovariectomy, ovariectomy + hPTH-1-37 treatment and ovariectomy + E_2 treatment on bone turnover. Results are displayed as means ± SEM (hPTH-1-37) N = 4 rats; all other groups N = 5 rats). Sham-operated rats treated with saline (NaCl) are compared to ovariectomized and saline (NaCl)-treated rats. The two remaining groups are compared to OVX control group (NaCl) (* $P < 0.05$ versus OVX NaCl; ANOVA).

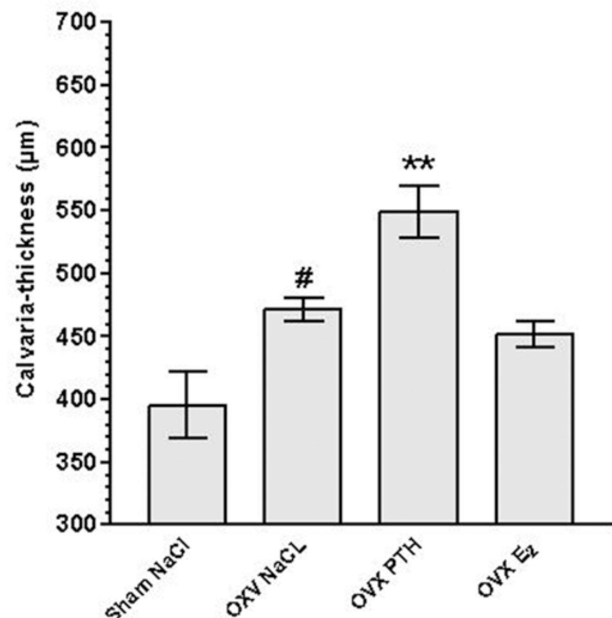


Fig. 6. Effects of sham operation, ovariectomy, ovariectomy + hPTH-1-37 treatment and ovariectomy + E_2 treatment on calvaria thickness. Results are shown as means ± SEM (hPTH-1-37) N = 4 rats; all other groups N = 5 rats). Sham-operated rats treated with saline (NaCl) are compared to ovariectomized and saline-treated rats. The two remaining groups are compared to OVX control group (NaCl) (# $P < 0{,}05$ versus Sham NaCl; ** $P < 0.01$ versus OVX NaCl; ANOVA).

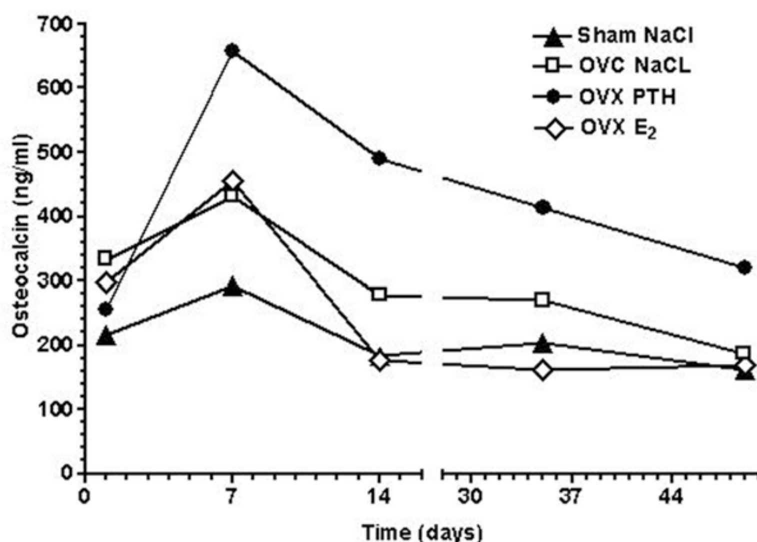


Fig. 7. Effects of sham operation, ovariectomy, ovariectomy + hPTH-1-37 treatment and ovariectomy + E_2 treatment on serum osteocalcin over the time of the experiment. Results are displayed as means ± SEM (hPTH-1-37) N = 4 rats; all other groups N = 5 rats). Sham-operated rats treated with saline (NaCl) are compared to ovariectomized and saline-treated rats. The two other groups are compared to the OVX control group (NaCl) (ANOVA).

Serum Osteocalcin

The serum concentration of osteocalcin, a marker of bone formation/turnover, was significantly increased in ovariectomized rats at all times (Sham NaCl versus OVX NaCl, $p < 0.05$; ANOVA). During the first week, E_2 treatment caused no alteration compared to the OVX NaCl control group. In the following, osteocalcin levels following E_2 decreased significantly (OVX NaCl versus OVX E_2, $p < 0.05$). After 7 days osteocalcin levels following hPTH-1-37 had reached a maximum and over time, hPTH-1-37 treatment continued to elicit increased osteocalcin levels (OVX NaCl versus OVX PTH, $p < 0.01$) (Fig. 7).

Discussion

Parathyroid hormone plays a pivotal role in the regulation of calcium homeostasis. Already as early as in the Twenties of the last century, the osteoanabolic effects of PTH after intermittent application have been observed for the first time [15]. During the Seventies, PTH was taken into consideration as a possible osteoanabolic drug for the treatment of postmenopausal osteoporosis [4]. Many studies were carried out to investigate the underlying mechanism and effects of PTH. In order to induce an osteoanabolic effect, PTH has to be applied intermittently, whereas continuous infusion of PTH causes catabolic processes, especially at the corticalis [16, 5, 2, 17]. The osteoanabolic effect of PTH in ovariectomized rats is primarily visible as an increase of trabecular thickness and cancellous bone volume of the long bones and vertebrae. This was demonstrated in several studies for young and adult ovariectomized rats, for male rats, as well as for rats with a chronic osteopenia [18, 19, 20, 21, 22, 23, 24]. The cancellous bone volume increases up to the level of the sham-operated control rats (see also Figs. 3 and 4) [25]. Destroyed trabeculae and trabecular connections are not rebuilt but the remaining tissue is strengthened [26]. PTH-1-34-treated bone gains a new stability which simultaneously increases bone strength and lowers the risk of fractures in the postmenopausal osteoporotic skeleton [23, 27]. Other studies show an increase in cortical thickness and a decrease of bone marrow space in ovariectomized rats [20, 28]. Furthermore, studies in postmenopausal women observe the capability of PTH to diminish the risk of fractures by strengthening the trabeculae [29, 30]. Even in younger women who are afflicted with GnRH-induced estrogen loss, PTH can prevent bone loss and increases bone mineral density [31, 32]. Regarding the catabolic effect of PTH, some studies demonstrate that PTH treatment does not affect the corticalis of women. However, the hypothesis that bone becomes more sensitive to the catabolic effects of PTH after estrogen loss and thus endogenous PTH causes osteoporosis could not be confirmed [33, 34, 35].

Whitfield et al. conclude that, after intermittent application of PTH, first a phase of osteoclast activation occurs [2]. Released calcium causes a positive feedback mechanism concerning osteoblast function and differentiation. Also, messengers like TGF-b, IGF-I and BMPs are released from the bone matrix, which supports the differentiation of the osteoblast precursor cells. Simultaneously released calcium interacts with osteoblast calcium receptors and thus promotes the activity of these cells. PTH activates PTH receptors of osteoblasts and pre-osteoblasts, thereby stimulating their differentiation. In addition, the production of proteins, which prevent osteoblasts from apoptosis, is induced and the lifespan of these cells is prolonged.

The net result of this cascade is an extensive differentiation of osteoblast precursors as well as an enhancement of natural osteoblast activity and causing an imbalance between bone resorption and formation, tending more to bone formation. A first stimulation of osteoclast activity by PTH-1-84 as well as by synthetic PTH-1-34 was not observed in experiments of Kimmel et al., using the model of the ovariectomized rat [10]. Until now it is unclear whether a first phase of osteoclast activation occurs or not. However, the net results of intermittent PTH are osteoanabolic due to an enormous increase in osteoblast numbers, demonstrated in studies of Liu and Kalu [25].

The present study clearly confirms these anabolic effects, using human PTH-1-37. Radiological evidence for an increase of bone density of *femur corticalis* and *facies poplitea* corresponds with a significant increase in femur density. Moreover, osteoanabolic effects were also observed at the calvaria in the form of a significant increase in calvarial thickness (Fig. 6). The highest bone turnover was detected in the PTH group (n = 4) which showed significantly increased space between tetracycline and calcein fluorescent labels (Fig. 5). The PTH group exhibited the highest values of serum osteocalcin during the whole period of the study (Fig. 7). An increase in osteocalcin values after PTH treatment was also observed by Liu et al. [19]. Both results indicate an enhanced osteoblast activity and bone turnover caused by treatment with hPTH-1-37. In this study, differential effects of hPTH-1-34 and hPTH-1-37 were examined *in vitro*. The results suggest that hPTH-1-37 may beneficially modify bone-turnover more so than hPTH-1-34. Inhibition of osteoclastic differentiation was more potently suppressed by hPTH-1-37 than by hPTH-1-34. In addition, early osteoblastic markers were more markedly elevated following hPTH-1-37. These studies do not provide conclusive insight into mechanisms of action that may lead to distinguishing properties of various PTH derivatives. It may be hypothesized that conformational differences between different-length derivatives could alter receptor binding and affinity as well as stability *in vitro* and *in vivo*.

In the investigated animal model of the ovariectomized rat hPTH-1-37 showed osteoanabolic effects in line with earlier findings using other PTH forms. Treatment with hPTH-1-37 resulted in a clear increase of bone mass compared to controls [10, 22, 36].

However, head-to-head *in vivo* studies, preferably in clinical trials, will have to be carried out to underline the importance of understanding differential effects of PTH derivatives and implications of such differences for clinical practice.

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