

Efficiency of 48h vs. 24h Injection of Parathyroid Hormone for Amelioration of Osteopenic Spine Properties in Male Rats

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Abstract: Daily application of parathyroid hormone (PTH 1-34) is used for treatment of osteoporosis. It was investigated whether orchietomy-induced osteoporotic changes in spine can be ameliorated by every 48h administration of PTH in aged male rats. Eight-month-old male Sprague-Dawley rats were sham operated (n=24) or orchietomized (Orx, n=36) and maintained untreated over 12 weeks. Thereafter, both tibia underwent transverse metaphyseal osteotomy (Komrakova et al. 2011, J Endocrinol; 209:9-19) and rats were divided into 5 groups treated s.c. as follows: 1) sham vehicle; 2) sham PTH every 24h (PTH/24h); 3) Orx vehicle; 4) Orx PTH/24h; 5) Orx PTH every 48h (PTH/48h). PTH dosage was 40 µg/kg BW per injection. After 5 weeks, lumbar vertebral bodies were used in computed tomographical, biomechanical, histomorphological, ashing and gene expression analyses. Cortical and trabecular densities, biomechanical properties, serum osteocalcin level increased significantly after PTH treatments in all groups (yield load, sham: 232±17N, sham PTH/24h: 376±12N, Orx: 239±16N, Orx PTH/24h: 324±31N, Orx PTH/48h: 297±17N). Bone inorganic weight enhanced after daily PTH application in Orx rats. Bone gene expression did not differ (P>0.05) among the groups. Both PTH administration regimes (24h and 48h) improved impaired bone structure in osteopenic rats. Every 48h application was less effective, however, it improved bone properties to the level observed in healthy (sham) rats. Considering limitation of daily treatments known in humans, these results may be useful for further clinical studies.

Keywords: Biomechanics, Gene expression, Lumbar spine, Osteoporosis, Parathyroid hormone.

INTRODUCTION

Evaluation of the lumbar vertebrae is essentially important in the treatment of osteoporosis and osteoporosis related fractures. Spine fractures lead to continuous back pain and spinal deformity that predicts further vertebral and non-vertebral fractures [1].

Hypogonadism or deprivation of androgen by surgical or chemical treatment in men can lead to developing of osteoporosis and increased fracture occurrence [2]. The mortality rate associated with fractures is higher in men than in women [3], though women are at greater risk of developing of osteoporosis. Statistical studies predicted that one out of 5 men aged over 50 would have an osteoporosis-related fracture [4] and this figure would rise [5]. Despite the statistics and the availability of treatment options, osteoporosis is still under-recognized in men, the treatment remains inadequate and the most studies are still conducted on female subjects [3, 6].

Currently, human parathyroid hormone (hPTH 1-84) and its teriparatide, recombinant hPTH 1-34, are being used as anabolic agents for treatment of severe osteoporosis in women and men [7]. The parathyroid hormone regulates serum calcium level and stimulates new bone formation when applied intermittently (by daily injections) [8]. The mechanisms by which intermittent administration produce anabolic effects are of considerable interest, however, they are not completely understood so far. Intermittent PTH administration directly activates survival signaling in osteoblasts, attenuates osteoblast apoptosis, thereby increasing osteoblast number, bone formation rate and bone mass, but does not affect osteoclast number [9].

PTH has been reported to increase spine bone mineral density (BMD) when applied at several dosages, for any duration, in different clinical situations and in combinations with other agents [10]. However, PTH treatment has significant limitations because of the high cost and daily subcutaneous administration [11]. In animal based studies, various regimes of PTH administration were used. However, the results obtained from these trials show a variation and it is difficult to reach a definitive conclusion [12, 13]. Recent study showed that PTH administration improved bone healing in both healthy and ovariectomy-induced osteopenic

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rats regardless of application frequency (7, 5 times per week or every other day) [14]. Part of the present study has been published previously [15, 16]. It was shown, that in aged males the effect of PTH on bone healing was less when it was applied with less frequency (every day vs. every other day) [15]. The proximal femur responded to PTH treatments positively showing improved biomechanical properties, mineral content and trabecular bone parameters [16]. Thus, PTH improved tibia healing and reversed structural impairment in femur in male rats. However, evaluation of the lumbar vertebral region is also extremely important. Moreover, various responses to PTH by different anatomic sites have been reported [10, 14, 17].

In the present study we investigated whether orchietomy-induced osteoporotic changes in spine can be ameliorated by administration of PTH every 48h in aged male rats. Comparisons were made with PTH applied at 24h interval.

MATERIAL AND METHODS

The animal study protocol was approved by the local regional government and conformed to the German animal protection laws.

Eight-month-old male Sprague-Dawley rats (Janvier, Le Genest-Saint-Isle, France) were bilaterally sham operated ($n = 24$) or orchietomized ($n = 36$, Orx) under i.p. ketamine and xylazine anesthesia (60 mg and 8 mg/kg of body weight (BW), respectively). After 12-week development of osteoporotic changes in bone, ketamine-xylazine-anesthetized rats underwent a transverse osteotomy of tibia metaphysis [18]. Data on osteotomy healing have been published previously [15].

PTH (1-34) treatments began on the next day after osteotomy and continued for 5 weeks. In group 1, sham-operated rats (sham) received vehicle (0.9% NaCl) every 24h. In group 2, sham rats were treated with PTH every 24h (sham PTH/24h). In group 3, Orx rats were given vehicle every 24h (Orx). In group 3 and 4, Orx rats were treated with PTH either every 24h or every 48h (Orx PTH/24h or Orx PTH/48h, respectively). A dosage of PTH was 40 $\mu\text{g}/\text{kg}$ BW per injection.

The labeling of new bone formation was done with 4 fluorochrome dyes applied: xylenol orange (90mg/kg BW) on day 13, calcein green (10 mg/kg BW) on day 18, alazarin complexone (30 mg/kg BW) on days 22 and 24 and tetracycline (25 mg/kg BW) on day 35 after beginning of treatments, respectively. All injections were done subcutaneously.

After 5 weeks of treatments, CO₂-anesthetized rats were decapitated. Immediately, the lumbar vertebral bodies were dissected free of soft tissues and spinal cord and stored at -20°C until further analyses. The sixth vertebral body (L6) was stored at -80°C for analyses of gene expression.

Flat-Panel Volume Computed Tomography (fpVCT)

The third vertebral body (L3) was scanned using an fpVC tomograph (GE Global research, Niskayuna, NY, USA). Body of vertebra was analyzed on an Advantage Workstation (Version 4.2, General Electric Healthy Care, Milwaukee, WI, USA). Processes, laminae and endplates

were not included in the analysis. Cortical and cancellous bone mineral densities were calculated using hydroxyapatite standards of several mineral densities [19].

Ashing

The second vertebral body (L2) was dried in a muffle oven at 110°C for 24h and then ashed at 750°C for 1h. Ash weight was expressed relative to cylindrical volume of vertebral body. Calcium content was determined using atomic absorption spectrometer (4100, PerkinElmer, Germany). Orthophosphate content was assayed by colometric method (spectral photometer DM4, Zeiss, Germany).

Biomechanical Analyses

The fourth vertebral body (L4) was tested biomechanically using testing device (Zwick/Roell, Ulm, Germany). The load was applied at the caudal end plate along the cranial-caudal axis of the vertebral body [20]. Stiffness, yield load and maximal compressive strength (Fmax) were determined.

Histomorphological Analyses

The entire first vertebral body (L1) was subjected to the sequential ascending concentrations of ethanol, embedded in methylmethacrylate and cut sagittally at a thickness of 150 μm using diamond saw microtome (Leica SP 1600, Leica Instruments GmbH, Nussloch, Germany). Fluorochrome labeled sections were analyzed qualitatively. Four central sections were microradiographed with the aid of faxitron (Cabinet X-ray system, Hewlett-Packard, Buffalo Grove, IL, USA) using KODAK Industrex film (100NIF, SR 45). The microradiographs (Fig. 1) were analyzed by QWin image analysis program (Leica, Bensheim, Germany) using a digital camera (Leica DC300F) and a zoom stereo microscope (Leica MZ75). The following cortical and trabecular parameters were measured: mean (ventral and dorsal) cortical width, cortical area and cortical density, endosteal trabecular density, number of trabecular nodes, number of nodes per mm² and trabecular width (Fig. 2).

Gene Expression Analyses

The sixth vertebral body (L6) was homogenized using micro-dismembrator S (Sartorius, Goettingen, Germany). Total RNA was extracted with the aid of RNeasy™ Mini Kit (Qiagen, Hilden, Germany) and assessed by a photometer (Biometra, Goettingen, Germany). RNA samples (100ng) were then reverse-transcribed using Superscript™ RNase H-reverse transcriptase (Promega, Mannheim, Germany). Expression of rat genes, alkaline phosphatase (Alp1), osteocalcin (Oc), insulin-like growth factor-1 (Igf-1), receptor activator of nuclear factor κB ligand (Rankl), osteoprotegerin (Opg) were determined by quantitative real-time polymerase chain reaction (qRT-PCR) based on SYBR Green detection using iCycler (CFX96, Bio-Rad Laboratories, Munich, Germany). Ready-to-use primer pairs were obtained from Qiagen (QuantiTect® Primer Assays, Hilden, Germany). Relative gene expression was calculated using 2^{- $\Delta\Delta\text{CT}$} method [21] for each gene of interest relative to the value observed in intact group. In the intact group ($n = 10$), male rats were intact non-orchietomized, non-osteotomized, untreated, maintained under the same conditions as the treatment groups, were of the same age and

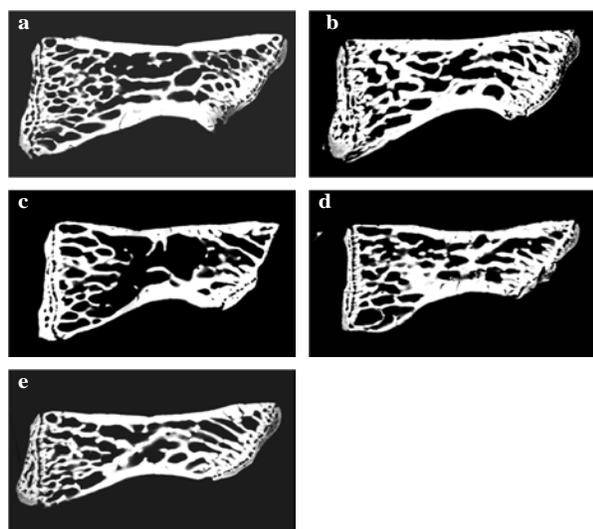


Fig. (1). Images of microradiographed sagittal sections of L1 made after 5-week treatments in groups: (a) sham, (b) sham PTH/24h, (c) Orx, (d) Orx PTH/24h, (e) Orx PTH/48h.

had a comparable body weight. Reference gene was β -2 microglobulin.

Serum Analyses

Serum analyses were conducted at the Department of Clinical Chemistry, University of Goettingen. Serum testosterone level was measured using liquid chromatography tandem mass spectrometry with the aid of a Quattro Premier XE Micromass-System (Waters). Serum osteocalcin (OC) was determined by electrochemiluminescence immunoassay analy-

ses and alkaline phosphatase (ALP) by colorimetric assay using automated chemistry analyzer (Roche/Hitachi Modular) and commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical Analyses

Analyses of variance (ANOVA, $P < 0.05$) were applied to reveal the effect of PTH on bone parameters (SAS 9.1; SAS Institute, Cary, NC). Differences between individual means were estimated using Schéffe-test ($P < 0.05$). Data are shown as means and standard error of the means (SEM). Kruskal-Wallis test and Dunn multiple comparison test were used for analyses of relative gene expression (GraphPad Prism 4.0; San Diego, CA).

RESULTS

Flat-Panel Volume Computed Tomography (fpVCT)

Cortical BMD increased significantly after PTH treatments in both sham and Orx groups. In “PTH/48h” group, cortical BMD was lower than in “PTH/24h” group, being however higher than in the untreated sham group (Table 1). Cancellous BMD did not change significantly among the treatment groups. The differences between sham and Orx rats were not revealed.

Ashing

Ash to volume ratio increased significantly after daily PTH administration in Orx rats (Table 1). Among other treatment groups, inorganic weight did not differ significantly. The molar ratio of calcium to orthophosphate did not change after PTH treatments.

Table 1. Analyses of Lumbar Vertebral Bodies (L) in Orchietomized (Orx) or Sham Rats Treated Either with PTH Every 24h (PTH/24h), Every 48h (PTH/48h) or given Vehicle During 5 Weeks. At Least 10 Replications Per Treatment Group were Done

	Sham		Sham PTH/24h		Orx		Orx PTH/24h		Orx PTH/48h	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
fpVCT (L3) mg/cm³										
Cortical BMD	463 ^a	4	510 ^c	4	451 ^a	3	495 ^b	8	477 ^a	4
Cancellous BMD	239	1	237	1	237	1	242	2	239	1
Ashing (L2)										
Ash/Volume (mg/mm ³)	1.09 ^{ab}	0.05	1.28 ^{bc}	0.12	1.02 ^a	0.07	1.36 ^c	0.11	1.16 ^{ac}	0.06
Ca ²⁺ /PO ₄ ³⁻	1.91	0.26	1.79	0.14	2.18	0.18	1.95	0.14	1.73	0.15
Biomechanics (L4)										
Stiffness (N/mm)	183	25	247	19	194	27	264	31	220	21
Yield load (N)	232 ^a	17	376 ^b	12	239 ^a	16	324 ^{bc}	31	297 ^c	17
Fmax (N)	252 ^a	17	378 ^b	11	254 ^a	13	338 ^{bc}	25	312 ^c	16
Histology (L1)										
Cortical width (mm)	0.40 ^a	0.02	0.52 ^b	0.02	0.40 ^a	0.01	0.49 ^b	0.03	0.50 ^b	0.02
Cortical area (mm)	7.3 ^a	0.6	10.7 ^c	0.2	6.9 ^a	0.3	8.7 ^b	0.5	9.4 ^b	0.3
Cortical density (%)	92 ^a	1	95 ^b	1	93 ^a	1	95 ^b	1	94 ^b	0.3
Trabecular density (%)	44 ^a	2	59 ^b	2	41 ^a	1	57 ^b	2	50 ^c	2
Trabecular nodes (n)	110 ^a	5	97 ^{ab}	4	87 ^{bc}	4	92 ^{bc}	4	82 ^c	4
Trabecular nodes/mm ²	4.4 ^a	0.3	3.9 ^{ac}	0.2	3.2 ^b	0.1	4.6 ^a	0.4	3.4 ^{bc}	0.1
Trabecular width (μm)	5.7 ^a	0.1	7.3 ^b	0.3	5.8 ^a	0.1	7.2 ^b	0.2	6.5 ^c	0.2

^{abcd} within row between treatment groups means with different superscripts differ ($P < 0.05$, Scheffé-test)

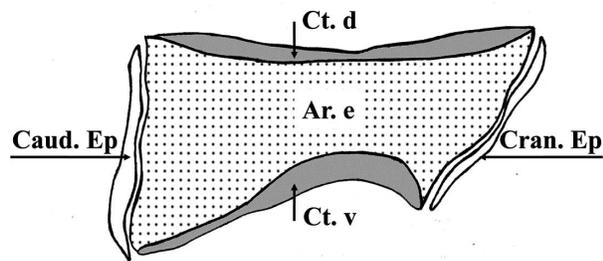


Fig. (2). Schematic diagram of measured cortical and trabecular bones of L1. Caud.Ep, caudal epiphysis and Cran.Ep, cranial epiphysis were not included in the analyses. Ct.d, cortical bone dorsal and Ct.v, cortical bone ventral were analyzed combined. Trabecular bone parameters were measured within endosteal area (Ar.e).

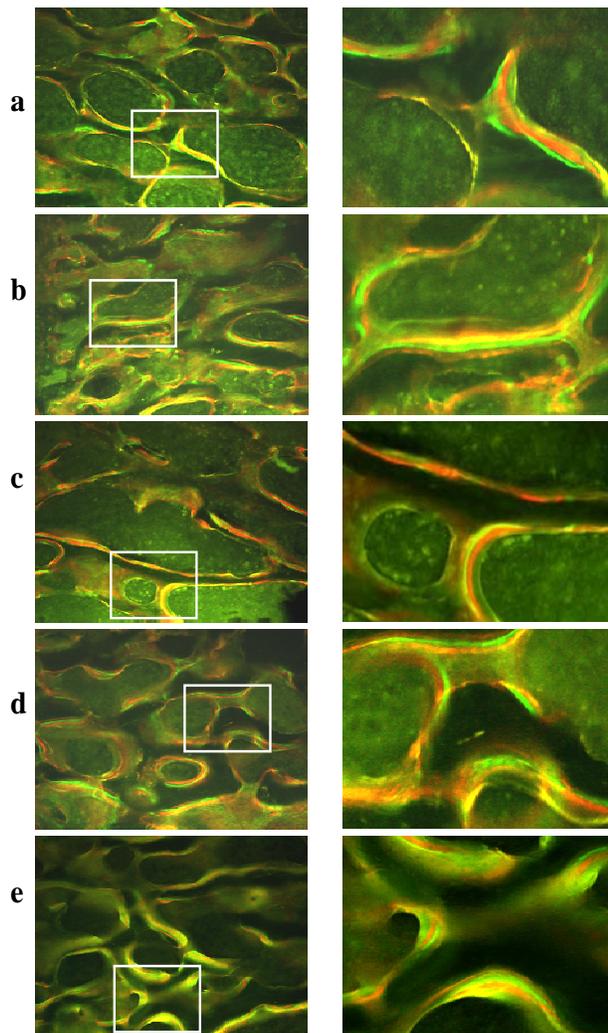


Fig. (3). Fluorochrome-labeled sections of L1 made after 5-week treatments in groups: within row (a) sham, (b) sham PTH/24h, (c) Orx, (d) Orx PTH/24h, (e) Orx PTH/48h. Rectangular frames within the first column indicate the region shown in the second column.

Biomechanical Analyses

Yield load and Fmax increased in Orx PTH/24h group to the enhanced level of sham rats treated with PTH (Table 1). In Orx PTH/48h group, the increase was less. However,

yield load and Fmax were at a higher level in this group than those measured in sham group. Stiffness did not differ between the groups. There was no difference in the biomechanical properties of L4 between Orx and sham rats.

Histological analyses

PTH treatment resulted in an increased trabecular density, thicker cortical and trabecular structures (Fig. 1 b,d,e). In Orx rats, trabecular network was less dense than in sham rats (Fig. 1 a,c). Qualitative analyses of fluorochrome labeled sections showed that both sham and Orx rats treated with PTH had thicker apposition bands and thereby more intense bone formation than untreated rats (Fig. 3).

Cortical width, area and density increased significantly after PTH treatment in all groups (Table 1), whereas, between Orx and sham rats, the differences were not found. Trabecular parameters improved after PTH administration in both sham and Orx group. The effect was less if PTH was applied every 48h. In Orx rats, number of trabecular nodes was lower compared to that observed in sham rats.

Gene Expression Analyses

There were no significant differences in the expression of bone genes in vertebral body among the treatment groups (Fig. 4). However, the tendency of Oc and Rankl gene expression to increase after PTH treatments was observed.

Serum Analyses

The serum testosterone significantly decreased to the level around zero in Orx rats (0.02 ng/mL, SEM=0.003), whereas in sham rats it was 1.5 ng/mL (SEM=0.2) on average. PTH treatments significantly enhanced OC level in all rats and did not change ($P>0.05$) ALP activity [15].

DISCUSSION

The intent of the present study was to investigate the effect of every 48h PTH (1-34) treatment on lumbar spine in osteopenic aged male rats and compare it with every 24h application of the teriparatide. Orchiectomized rats display deleterious changes in bone within 4 weeks after orchiectomy and may be used for osteoporotic studies [22, 23].

In the present study, it was shown that every 24h treatment with PTH improved cortical and trabecular densities, enlarged cortical area and trabecular width, enhanced biomechanical properties and inorganic weight of lumbar spine in both Orx and sham rats. Daily PTH treatment has been reported to have positive effect on both trabecular and cortical bone in osteoporotic subjects [24].

The administration of PTH every 48h in Orx rats had beneficial effect on vertebral spine too, however, to a lesser extent. Nevertheless, bone properties were improved to the level observed in healthy rats. Similar regime-depending effect has been reported on the healing of tibia osteotomy as well as serum osteocalcin level in these rats [15] and on vertebral body in ovariectomized female rats of 6 months of age [17]. Interestingly, in the latter, PTH improved osteotomy healing irrespectively of administration regime [17]. Variations in bone mineral density (BMD) and bone disorder related to osteoporosis are largely determined genetically, however, the mechanisms of gene action are not

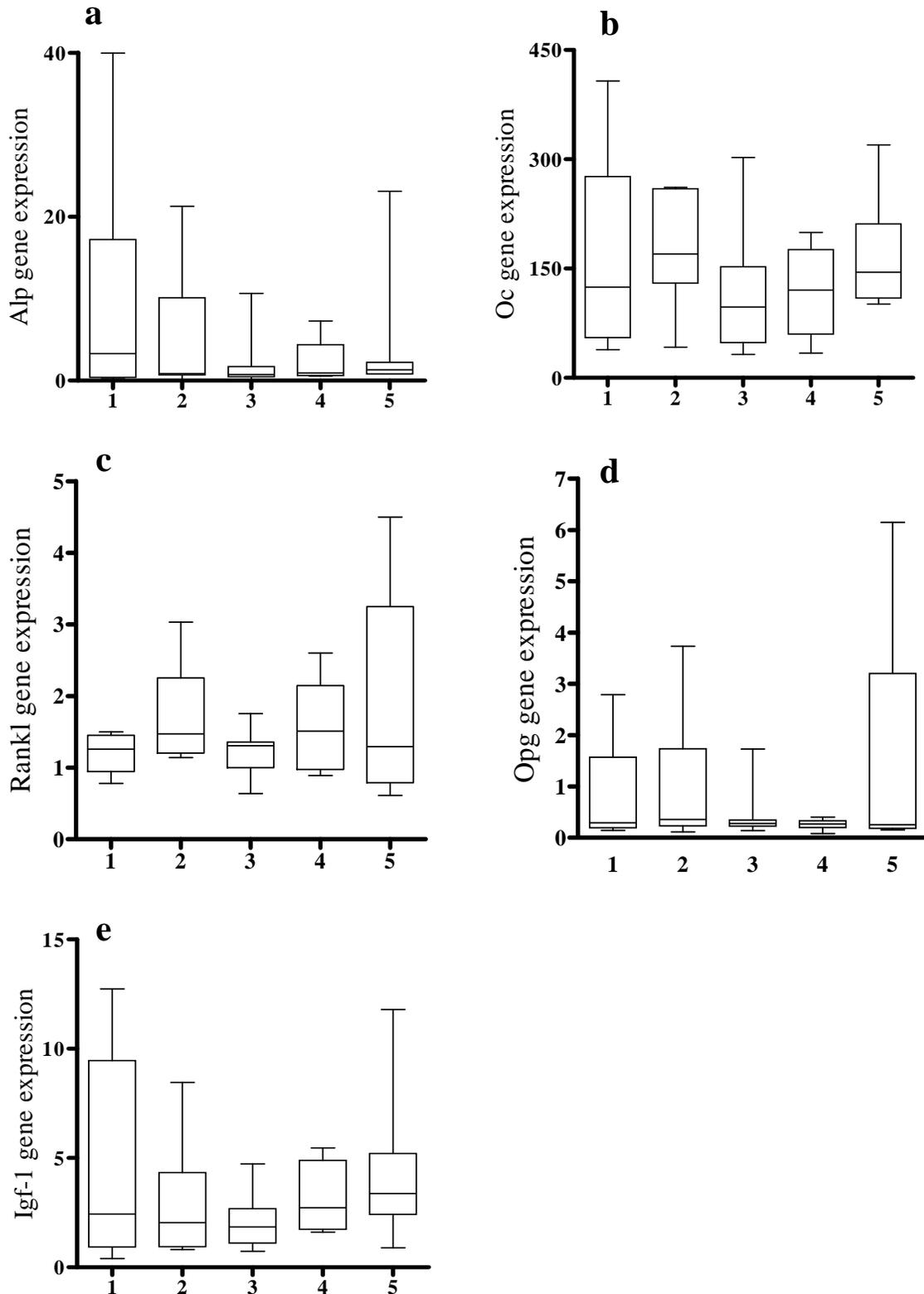


Fig. (4). Box plot illustrating relative mRNA expression level of (a) Alp, (b) Oc, (c) Rankl (d) Opg, (e) Igf-1 genes in L6 calculated using $2^{-\Delta\Delta CT}$ method relative to gene expression in untreated rats taken as 1. On the X-axis treatment groups: (1) sham, (2) sham PTH/24h, (3) Orx, (4) Orx PTH/24h, (5) Orx PTH/48h. At least 8 replications per treatment group were done. The differences were not significant ($P > 0.05$, Dunn test).

established so far [25]. The PTH administration slightly, though not significantly enhanced bone formation and resorption genes in lumbar spine in the present study, and

correspondently caused significant up-regulation of Igf-1, Opg and Rankl genes in the callus of tibia in these rats [15]. On the contrary, in ovariectomized (Ovx) rats no changes

were found in mRNA gene expression in the callus [14], whereas in lumbar vertebral body, bone genes were up-regulated after PTH treatment [17]. In the present study, PTH effect on spine was not blunt by orchietomy, being similar effective in both sham and Orx rats, whereas, stimulation of tibia healing by PTH was more efficient in sham rats [15]. Turner *et al.* [23] reported that gonadal hormones exerted distinct effects on bone metabolism in male and female rats. In OvX rats, loss of cancellous bone results from increased osteoclast-mediated bone resorption, whereas diminished bone formation is at least a contributing factor in Orx rats [23]. Various responses to PTH by different anatomic sites have been reported [10]. Additionally, the differences in age of the animals may contribute to the observed PTH effect.

Applying fpVCT analyses, the improved mineral density was detected solely in cortical bone after PTH treatments, whereas in trabecular bones this was not seen. However, the histomorphological analysis showed the significantly enhanced trabecular density in all PTH treated rats. This indicated that computed tomographical analysis could not adequately reflect important structural improvements that enhanced the biomechanical stability of bone tissue in these rats. The micro-computed tomography which provides high-resolution assessments of trabecular microstructure could be a more effective means for analyses of bone tissue in rat model.

Studying the effect of orchietomy on bone, the developing of osteopenia was confirmed. Histological analyses revealed reduced trabecular number in Orx rats. However, cortical bone parameters were less affected by androgen deficiency. It is known that cancellous bone is more affected by gonadal hormone depletion than cortical bone [22, 23]. This may explain unaltered biomechanical properties of lumbar vertebral body in Orx rats.

Concluding, every 24h PTH administration improved the osteopenic bone already after 5 weeks so that the level of sham, i.e. healthy group was achieved and in most cases even surpassed. The every 48h application resulted in significant improvement of cortical structure, biomechanical parameters and trabecular density in comparison to the untreated sham group. In sham group, PTH affected bone also anabolically. Considering limitations of daily treatment and possible side effects [11], the application of PTH with less frequency (e.g. every 48h) may serve as an option for osteoporosis treatment in male subjects. The comparisons with other studies showed that the effectiveness of PTH treatment is dependent on age and hormonal status of the subjects; different anatomic sites responded variously to PTH treatment. These indications may be useful in perspective for further clinical studies conducted in osteoporotic patients.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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