



PTH₁₋₃₄ improves devitalized allogeneic bone graft healing in a murine femoral critical size defect[☆]

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ABSTRACT

The treatment of large segmental defects of long bones resulting from trauma, infection, or bone tumor resections is a major challenge for orthopedic surgeons. The reconstruction of bone defects with acellular allografts can be used as an osteoconductive approach. However, devitalized allografts are associated with high rates of clinical failure as a result of poor intrinsic osteoinduction properties and a lack of further remodeling. Nevertheless, evidence suggests that due to its anabolic properties, teriparatide (PTH₁₋₃₄) could be effective as an adjuvant therapy for massive allograft healing. Therefore, our goal was to investigate in a murine critical-sized defect model whether the intermittent administration of PTH₁₋₃₄ improves the incorporation and revitalization of acellular structural bone allografts. Thus, a 2.5-mm critical-sized defect was established in the right femur of C57BL/6 mice, followed by the reconstruction with a devitalized cortical structural allograft. A titanium micro locking plate was applied to the anterior femoral surface and secured in place with self-tapping locking screws. Subsequently, daily doses of PTH₁₋₃₄ (30, and 40 µg/kg) or saline were administered to the mice for 14 days after surgery. The mice were maintained without PTH₁₋₃₄ therapy for an additional 7 days before being euthanized at 3 weeks post-surgery. Bone graft consolidation was assessed on radiographic images and by histomorphometric analysis. Additionally, to determine the frequency of osteoprogenitor cells in the bone marrow and their *in vitro* osteogenic capacity, stromal cells were isolated from the bone marrow of animals treated with 30 or 40 µg/kg/day of PTH₁₋₃₄ following the same protocol used for the experimental animals. Our results suggest that intermittent PTH₁₋₃₄ treatment at 30 µg/kg/day after femoral allograft reconstruction surgery accelerated the healing process as evidenced by new bone formation induced on endosteal and periosteal surfaces, enhanced revitalization of allogeneic graft, and increased frequency and osteogenic capacity of bone marrow stromal cells (BMSC). These findings should encourage further studies aimed at investigating the potential therapeutic use of intermittent PTH₁₋₃₄, specifically with regards to the optimal dosing regimen in clinically challenging orthopedic scenarios.

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Introduction

Large skeletal defects with significant bone loss are commonly associated with high-energy trauma, surgical resections of bone tu-

mors, infection, and osteomyelitis [1]. Despite the recent advances and refinements in surgical techniques, the reconstruction of extensive bone loss remains a major challenge for the orthopedic surgeon [2]. While small bone defects up to 5 cm can be treated with conventional cancellous bone grafts or bone substitutes [3], larger segmental (or critical-sized) bone defects need a more complex specialized approach [2,4]. In this particular setting, gold standard treatment with bone autografts to accelerate bone regeneration is associated with several disadvantages, including donor site morbidity, limited supply, and infection [5,6].

Allografts are an alternative to autografts for bone grafting procedures as they support bone formation due to their osteoconduc-

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tive properties, are available in less limited supply, and are associated with less morbidity since they do not require an additional surgical procedure [7,8]. However, bone allografts present several limitations, such as incomplete transplant integration, graft devitalization, and resorption leading to decreased mechanical stability and predisposing them to structural failure [2,7,9,10]. Strategies for managing this limitation, such as osteoinductive approaches including the delivery of local growth factors like bone morphogenetic protein or platelet-rich plasma, have been considered [11].

Parathyroid hormone (PTH) is a polypeptide hormone with 84 amino acids (1–84), which is synthesized by the parathyroid glands and is released through circadian dynamics or in random pulses. PTH maintains serum calcium levels in the normal range via action in the main target organs (bones and kidneys) and indirectly in the digestive tract, thereby facilitating the activation of vitamin D [12]. This control of blood calcium levels is performed through a negative feedback mechanism, where the secretion and release of PTH by the parathyroid is controlled by the serum levels of $[Ca^{2+}]$ [13,14]. In contrast, intermittent or pulsatile doses of an active recombinant human peptide sequence of parathyroid hormone (teriparatide) exert an anabolic effect on bone due to the increase in osteoblastogenesis and/or by the decrease in the rates of osteoblastic apoptosis [14,15]. The increase in osteoblastogenesis, in turn, results from the increase in osteoblastic differentiation and, to a lesser extent, from the increase in cell proliferation. Thus, the anabolic action of PTH takes place by controlling the progression of osteoblasts in the cell cycle, which is diverted to enter the path of differentiation for the acquisition of the differentiated osteogenic phenotype. Regardless of the action on the cell cycle, PTH also directly stimulates osteoblastic differentiation [16].

For the treatment of osteoporosis, intermittent teriparatide administration acts as an anabolic agent, increasing the rate of bone formation, which, by predominating over resorption, generates a positive balance in favor of bone formation, and consequently, an increase in bone mass [17,18]. With the advances regarding the knowledge of the anabolic properties of intermittent PTH_{1–34} administrations, its systemic use in several orthopedic conditions is being increased, particularly in situations in which the bone repair process is defective [19–21]. The rationale of the systemic approach in the orthopedic setting is to make the host environment more favorable to promote and accelerate the host's intrinsic ability to regenerate bone [22]. Hence, our goal was to investigate whether the intermittent administration of teriparatide (PTH_{1–34}) improves the incorporation of devitalized bone allografts in a critical-sized bone defect with stable fixation in a mouse model. We also explored the frequency and the osteogenic potential of mice BMSC after PTH_{1–34} administrations.

Materials and methods

Experimental animals

Twenty-six 13–16 week-old male C57BL/6 mice weighting 20–23 g were purchased from Granja R.G. (São Paulo, Brazil) and housed in a HEPA filtered rack (ALESCO®, São Paulo, Brazil) under 12-hour light and dark cycles with free access to water and rodent diet. All the experimental procedures performed in this study were approved by the animal care committee of the National Institute of Traumatology and Orthopedics (Protocol CEUA INTO 002/2014).

Anesthesia and postoperative management

All mice were given general anesthesia consisting of 100 mg/kg ketamine and 10 mg/kg xylazine by an intraperitoneal injection. Ketoprofen 1% (2 mg/kg/day) was administered subcutaneously one

day before and after surgery for two successive days as pain control. To avoid surgical site infections, all animals were given a perioperative single dose of prophylactic antibiotics (clindamycin 50 mg/kg subcutaneously).

Femoral allograft reconstruction surgery

A lateral incision was made from the right hind limb trochanter to the lateral femoral condyle to expose the femur shaft using a hemostat-curved forceps (Fig. 1). A pure titanium (TiCp) micro locking plate (10 mm long and 1.5 mm wide; MouseFix Plate XL; RISystem AG, Davos, Switzerland) with a cylindrical undersurface and self-tapping micro screws was applied on the anterior femoral surface. Next, two holes were drilled at positions 1 and 6 using a 30G needle and two TAN self-tapping locking screws (2 mm long, 0.47 mm outer diameter, weight: 5 mg; MouseFix Screw 2 mm; RISystem AG) were inserted and locked to secure the plate. After drilling, two other screws were inserted at positions 2 and 5. Positions 3 and 4 were left empty (Fig. 2). A 2.5-mm long mid-diaphyseal femoral osteotomy was performed using a 0.22 mm diameter Gigli saw (RISystem AG) for the establishment of a segmental bone defect.

The defect was filled with 2.5-mm devitalized allografts previously obtained from donor femurs of C57BL/6 mice that were the same age as the experimental animals. Briefly, the preparation of allografts included a sequence of flushing of the bone marrow with saline and storage in sterile conditions at -80°C for at least 24 h before use. A few minutes before surgery, the grafts were thawed in saline at room temperature and implanted into the mid-diaphysis defect. The allografts were secured in place with a plate and screws and additional cerclage with suture thread (Vicryl 4-0, Ethicon®, Johnson & Johnson, NJ, USA). As proof that spontaneous consolidation of the defect would not occur, the same osteosynthesis was performed in two animals without the reconstruction of the gap with the allogeneic graft. After surgery mice were housed individually and allowed unrestricted weight bearing.

Administration of PTH_{1–34}

All mice submitted to allograft surgery in the mid-diaphysis of the right femur were treated with daily doses (30 and 40 $\mu\text{g/kg}$) of PTH_{1–34} (Forteo®, Eli Lilly and Company, IN, USA) or saline, starting one day after the surgery, 7 days/week for 2 weeks.

The mice were maintained without PTH_{1–34} therapy or saline administration for an additional 7 days before being euthanized at the end of the third week post-surgery with an overdose of ketamine and xylazine (three times the anesthetic dose). Immediately after euthanasia, the grafted femoral bones were excised and processed for histological analysis. The initial sample consisting of 24 animals that underwent allograft reconstruction surgery was reduced to 20 animals, as one animal from the 30 $\mu\text{g/kg}$ PTH_{1–34} group was excluded due to complete graft resorption, while three animals from the saline group were excluded due to post-operative death. The latter 3 animals developed significant reduction in activity and food intake during the experimental period that resulted in impaired general health conditions and were thus euthanized (Supplementary Figure 1).

Radiographic assessment of allograft healing

Representative radiographic images of reconstructed femoral defects were taken immediately after the surgery to assess the defect size, the anatomical alignment of the graft, and the stability of the osteosynthesis. Defect size was measured from radiographs in Digital Imaging and Communication in Medicine (mDicom) software format version 3.0.0 using the crosshair tool. A final radio-

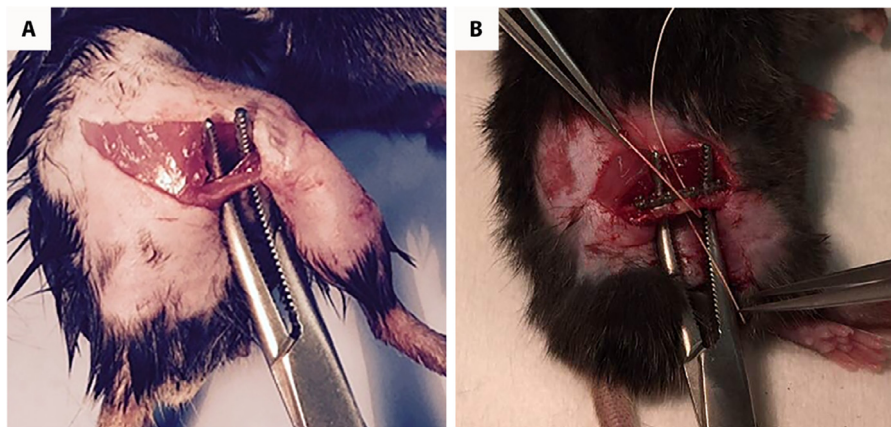


Fig. 1. Surgical procedure for femoral allograft reconstruction. (A) Lateral approach performed from the trochanter to the lateral femoral condyle and exposure of the femoral diaphysis using a hemostat-curved forceps. (B) A 2.5-mm long mid-diaphyseal femoral osteotomy was performed using a 0.22 mm diameter Gigli saw for the establishment of a segmental bone defect. The defect was filled with allogeneic devitalized bone graft and secured in place with a plate and self-tapping microscrews and additional cerclage with suture thread.

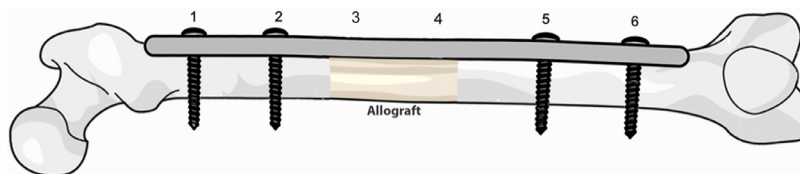


Fig. 2. Schematic presentation of the micro locking plate applied on the anterior femoral surface and secured in place with self-tapping locking screws at positions 1, 2, 5 and 6. Positions 3 and 4 were left empty.

graphic assessment was performed on the day of euthanasia (day 21).

Histological and histomorphometric analyses

After euthanasia, the femurs were dissected and fixed in 10% buffered formalin (pH 7.4) for 24 h. For routine histology, bone specimens were decalcified in 10% nitric acid overnight before being embedded in paraffin. Sections with thicknesses of 4 μ m were stained with hematoxylin-eosin (H&E) and evaluated microscopically using Aperio ImageScope version 12.3.3 (Leica Biosystems Inc., Lincolnshire, IL, USA).

Graft-host integration was determined when new bone formation originating from the endosteal and/or periosteal surfaces provided the union between the graft and the host bone. New bone formation was also quantitated at each graft-host junction in a pre-established region of interest (ROI) measuring 1 mm² defined at each junction. Due to technical issues with tissue sectioning, some cortical interfaces could not be evaluated. The quantitative assessment of new bone formation within the graft (revitalization) was performed using the Image J software (<https://imagej.nih.gov/ij/>) freehand selection tool and was expressed as the percentage of new bone formation as a fraction of the total area of the graft cavity.

Isolation and culture conditions for mouse bone marrow stromal cells (BMSC)

For *in vitro* experiments, another set of six 13–16 week-old male C57BL/6 mice were injected with daily doses of PTH_{1–34} (30 and 40 μ g/kg) or saline for 7 days via subcutaneous injections. One week after the end of the treatment (day 14), all mice were euthanized by an overdose of ketamine and xylazine (3 times the anesthetic dose). The hind limbs were collected and after removal of all soft tissue, the tibial and femoral marrow cavities were exposed af-

ter careful epiphyseal sectioning. Cells from the bone marrow were harvested by flushing the diaphysis with phosphate-buffered saline (PBS) supplemented with 0.5% fetal bovine serum (FBS, Gibco, Grand Island, NY) using a 10 mL syringe with a 21G needle. The eluted content was filtered through a 70 μ m cell strainer, washed with PBS, centrifuged at 300 g for 10 minutes, and suspended in Dulbecco's modified Essential Medium (DMEM low-glucose, Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (P/S, Gibco). The number of nucleated cells in the marrow suspensions were counted and plated according to the specific protocol of each *in vitro* assay (Fig. 3).

Colony-Forming Efficiency (CFE) Assay

Colony-Forming Efficiency (CFE) assays were carried out to quantify colony-forming units (CFU-Fs) as a measure of BMSC frequency in the marrow suspensions. Cells were seeded in triplicate in a 25 cm² flask at a density of 2×10^5 cells/cm² in 5 mL of DMEM supplemented with 10% FBS. After 3 days of incubation at 37 °C in 5% CO₂, the non-adherent hematopoietic cells were removed, and the medium was changed. On day 14, the colonies were fixed with 4% paraformaldehyde for 15 minutes and stained with 1% crystal violet (Sigma-Aldrich). Colonies with more than 50 cells were counted, and the Colony-Forming Efficiency (CFE) was expressed as the mean colony number relative to 2×10^5 bone marrow nucleated cells plated. Colony diameter was measured between the furthest two points that lay on the colony periphery and expressed in cm as an estimate of the proliferative potential of colonies.

In vitro osteogenic differentiation and von Kossa staining

To discriminate the osteogenic capacity of osteoprogenitor cells from the bone marrow (BMSC), cells were plated at a density of 2×10^4 /cm² in 24-well plates in DMEM supplemented with 10%

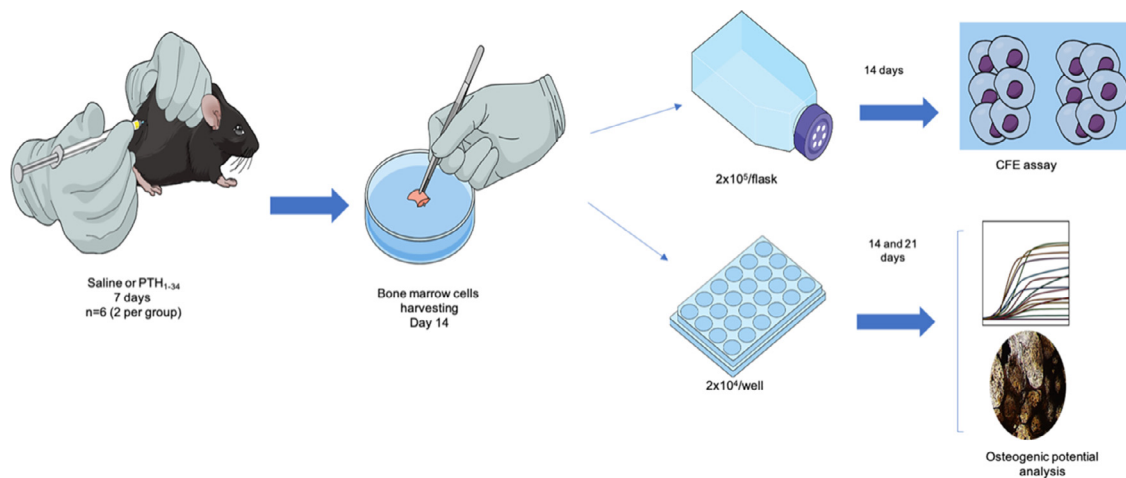


Fig. 3. Schematic diagram of *in vitro* experimental design after *in vivo* administration of PTH₁₋₃₄ ($n = 4$) or saline ($n = 2$) to C57BL/6 mice.

Table 1
Target genes and primers.

Target gene	Acession Number	Sequence (5'- 3')	Product (bp)
Osteocalcin	NM_199173.6	forward	AGC CTT TGT GTC CAA GCA G
		reverse	AGC TCA CAC ACC TCC CTC CT
Osterix	NM_001300837.2	forward	GAC ACA CCC CTA TGG CTC CT
		reverse	ACC TAG CCA GTT GCC TCC AG
Runx-2	NM_001278478.2	forward	CCT TGA CCA TAA CCG TCT TCA
		reverse	ATG CGC CCT AAA TCA CTG AG
β -actin	NM_001101.5	forward	CAT GTA CGT TGC TAT CCA GGC
		reverse	CTC CTT AAT GTC ACG CAC GAT

FBS and allowed to grow to total confluence. Osteogenic differentiation was induced by incubation with DMEM 10% FBS and antibiotics, containing 10 mM β -glycerophosphate, 5 mg/mL ascorbic acid 2-phosphate, and 10^{-6} M dexamethasone (all from Sigma) for 21 days, with medium change every 3 days. For the von Kossa staining, cell monolayers were fixed with 4% paraformaldehyde for 15 minutes at room temperature and incubated with 2% silver nitrate aqueous solution for 40 minutes, protected from light. Cells were washed 3 times with distilled water and exposed to UV light for up to 10 minutes. The quantification of mineralized areas was performed in 15 random microscopic fields using the Image J software.

Gene expression analysis

Osteogenic differentiation of BMSC after PTH₁₋₃₄ treatment was assessed by measuring the expression of osteoblast-specific genes *Runx2*, *Osteocalcin* and *Osterix* by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total mRNA was extracted from cells on days 14 and 21 of osteogenic induction using the TRIzol Reagent (Invitrogen, NY) according to the manufacturer's instructions. For cDNA synthesis, 1 μ g total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies). Signals were detected using the SYBR Green MasterMix (Thermo Fisher Scientific, UK) with gene-specific primers of *Runx2*, *osteocalcin* and *osterix*, and β -actin as a housekeeping gene (Table 1). Real-time reactions were performed in triplicate using a Line Gene 9600 Real-Time thermocycler (Hangzhou Bioer Technology, Binjiang, China). Relative quantification was performed using the Delta-Delta Ct method.

Statistical analysis

Data were expressed as mean $\pm$ standard error of mean (SEM) or standard deviation (SD), when indicated. Multiple comparisons

between groups were carried out using one-way ANOVA followed by Tukey's test (defect size, CFE assay, and gene expression analysis), Kruskal-Wallis (histological measurements), post-hoc Dunn's test (von Kossa staining) or the Chi-Square test (healed graft-host junctions) to assess differences among PTH₁₋₃₄ treatment groups and compared to the control groups, respectively. The significance level of 0.05 was applied for all analyses. All statistical analysis was performed by using Prism software (GraphPad software version 8.2.1, La Jolla, CA, USA).

Results

Allograft reconstruction surgery and clinical follow-up

The surgical procedure lasted 50–60 minutes on average from anesthetic induction to wound closure. In the first postoperative day, the animals showed a slight reduction in capacity when using the operated limb. After one week, the gait pattern was normalized and their performance was the same as observed before surgery. No animal showed postoperative wound complications or local/systemic reactions related to daily treatment with PTH₁₋₃₄ or saline.

The analysis of radiographs taken immediately after femoral allograft reconstruction surgery confirmed that the mean size of the diaphyseal defect was similar among the experimental groups 30 and 40 μ g/kg/day PTH₁₋₃₄ and untreated saline control (2.7 ± 0.4 mm, 3.1 ± 0.5 mm and 2.5 ± 0.4 mm respectively, $p = 0.647$) (Fig. 4).

Radiographic assessment of PTH₁₋₃₄ on allograft healing

On day 1 after surgery, all animals included in the study had stable osteosynthesis. In the final assessment on the day of euthanasia (day 21), the radiographic findings of the bone union

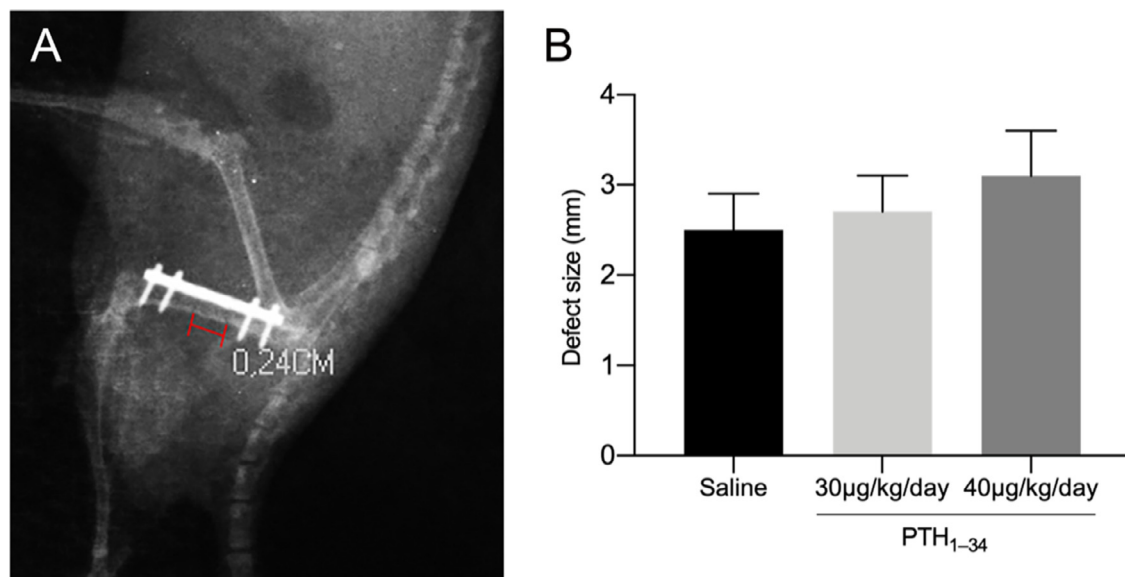


Fig. 4. Femoral defect size. (A) Measurement of the mid-diaphysis defect in lateral view radiographs taken immediately after femoral allograft reconstruction surgery. (B) Average size of the bone defect (mean $\pm$ SEM), $p = 0.647$.

were equivalent among animals from all groups. The graft interfaces with the host bone, identified on day 1 by radiotransparent lines (gaps), were no longer observed. The grafts were anatomically aligned in the mid-diaphysis of the femur and the fixation remained stable in all animals throughout the study period. In the two animals in which no graft was used to reconstruct the bone defect, the defect site remained empty until the end of the experiment (Fig. 5).

Histological assessment of allograft healing

Graft-host integration was similar between the 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ and saline groups. Osseous bridging was observed in 18 out of 18 allograft-host bone junctions in the control group and in 22 out of 22 in the 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ experimental group. In contrast, non-union rates at the graft-host interface were significantly greater in 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ treated animals (11 out of 30) compared to the 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ and to the untreated saline group ($p = 0.0002$) (Fig. 6).

The quantitative assessment of bone formation at the graft-host junction showed a greater volume of newly formed bone in 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ compared to 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ ($0.06 \pm 0.005 \text{ mm}^3$ and $0.045 \pm 0.005 \text{ mm}^3$ respectively, $p = 0.02$) and to untreated controls ($0.043 \pm 0.004 \text{ mm}^3$, $p = 0.03$). In animals treated with intermittent 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ bone neof ormation in the contact zones often showed extension to the endosteal and periosteal surfaces, while in 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ and in saline-treated animals the bone formation was limited to the contact zone between the cortices at the graft-host junction (Fig. 7). Non-union was observed when the bone defects were left empty (Supplementary Figure 2).

Histomorphometric assessment of allograft revitalization

Intermittent daily treatment with 30 $\mu\text{g/kg}$ PTH₁₋₃₄ for two weeks significantly increased the revitalization of allogeneic grafts compared to 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ ($29.7\% \pm 3.8\%$ vs. $18.3\% \pm 2.3\%$, $p = 0.03$) and to saline-treated controls ($15.3\% \pm 2.6\%$, $p = 0.01$). Furthermore, the lower 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ dose regimen, in addition to promoting the filling of allograft throughout the marrow cavity, reproduced the sponge-like aspect of cancellous bone and

the microenvironment of the medullary cavity, including repopulation with hematopoietic bone marrow. In contrast, 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ treatment resulted in scanty new bone formation that was insufficient to promote continuous host-graft integration. Similarly, in the saline-treated controls, the inner portion of the grafts was mostly filled with loose vascularized connective tissue lacking evidence of osteogenic activity (Fig. 8).

Frequency and osteogenic capacity of BMSC after PTH₁₋₃₄ administrations

CFU-F frequency was significantly higher in the bone marrow from mice injected with 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ (range, 26.30 to 28 per 2×10^5 nucleated cells, averaging 27.15 ± 1.2) compared to 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ (range, 6 to 9 per 2×10^5 nucleated cells, averaging 7.5 ± 2.12 , $p = 0.03$). There was no significant difference in the number of CFU-Fs from either treatment group nor from the untreated saline control animals (range, 12 to 21 per 2×10^5 nucleated cells, averaging 16.5 ± 6.3). Likewise, there was no significant difference in the colony diameter between the PTH₁₋₃₄ treatment groups and the untreated saline control indicating that proliferative potential of cells was not affected by intermittent PTH₁₋₃₄ treatment. Although in all groups the induced BMSCs were able to differentiate into osteoblasts as shown by the deposition of mineralized nodules with positive Von Kossa staining, a significant increase in nodular staining was observed in the 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ group (34.32 ± 1.72 , $p = 0.01$) compared to 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ (18.83 ± 8.04) and saline group (27.53 ± 4.32) (Fig. 9).

To gain further insight into the molecular mechanism underlying PTH₁₋₃₄ effects on allograft healing, we assessed the expression level of genes related to osteoblastic differentiation following *in vitro* induction of osteogenic differentiation for 14 or 21 days. The expression of *Osterix* increased significantly in the 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ group on day 21 compared to day 14 ($p = 0.03$) and was upregulated in comparison to both the 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ and saline groups ($p < 0.05$). Also, on day 21 *Osteocalcin* was overexpressed on 30 $\mu\text{g/kg/day}$ PTH₁₋₃₄ when compared to 40 $\mu\text{g/kg/day}$ PTH₁₋₃₄ ($p = 0.08$) and untreated saline ($p = 0.06$), though without reaching statistical significance. Intermittent PTH₁₋₃₄ treatment had no significant effects on *Runx2* expression. Changes in m-RNA expression of *Runx2*, *Osteocalcin* and *Osterix* are shown in Fig. 9.

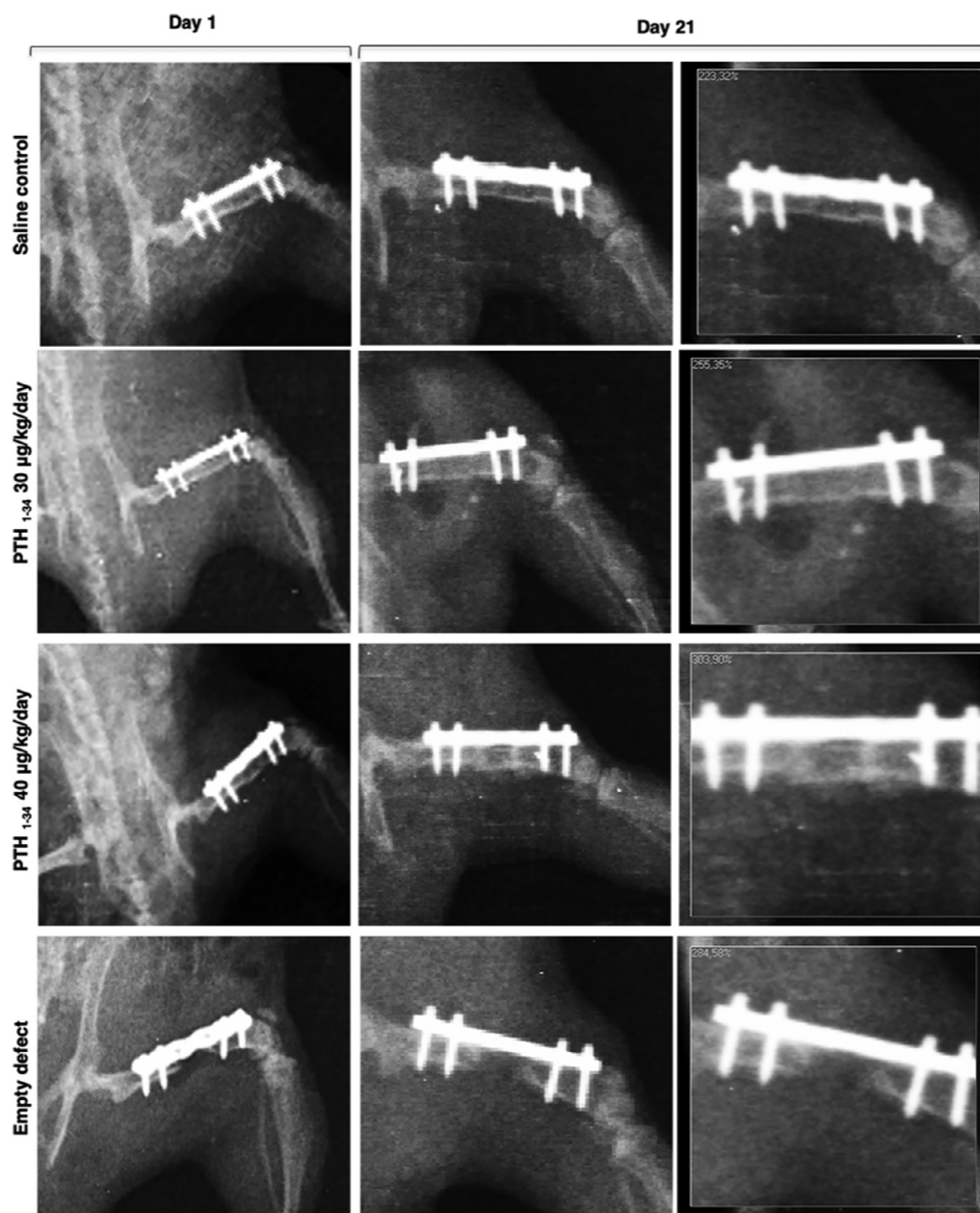


Fig. 5. Representative radiographic images of reconstructed femoral defects immediately after the surgery (left panel) and at the end of the experiment (center panel). The right panel shows a view in greater magnification of the same image displayed in the center panel. On day 21 graft-host integration was equivalent among animals from all groups. The grafts were anatomically aligned in the mid-diaphysis of the femur and the implant fixation remained stable in all animals throughout the study period. In two animals in which allograft reconstruction was not performed, the defect site remained empty (lower panel).

Discussion

In humans, teriparatide (PTH₁₋₃₄) is indicated for the treatment of osteoporosis, as it promotes an increase in bone mass and reduces the rates of low-impact osteoporotic fractures [23–25]. Over the last decade an increasing number of reports have proposed expanding the use of PTH₁₋₃₄ as an adjuvant therapeutic option to accelerate fracture healing [26–30], as an adjunct to treat nonunion [31–34], and to improve periprosthetic bone mineral density after total knee [35] or shoulder arthroplasty [36]. However, there are no reports that attest to the potentiating action of PTH₁₋₃₄ on osteogenesis and bone formation in a more challenging clinical scenario such as extensive long bone losses.

Although structural grafts are the main option for reconstructing large bone losses, their use is associated with high failure rates

[7,37,38]. This is largely imputed to the absence of osteogenic potential, which results in low capacity for allograft consolidation and revitalization. In animals, it was shown that strategies using systemic treatments with PTH₁₋₃₄, can reduce the biological limitations of devitalized allografts [39,40]. If confirmed in humans, these results have an important clinical significance, as they might result in increased bone healing and early graft-host integration.

Despite the limitations to reproduce the size of a segmental defect and human biomechanics in a small animal, the proof of the osteoanabolic action of PTH₁₋₃₄ in humans is still highly dependent on results obtained in animal models, in particular the cellular and molecular mechanisms that regulate bone healing [41]. The pioneering reports of Andreassen et al. (1999) and Holzer et al. (1999) showed that PTH₁₋₃₄ provided increased bone formation and callus quality in a model of tibial fracture in rats [42,43].

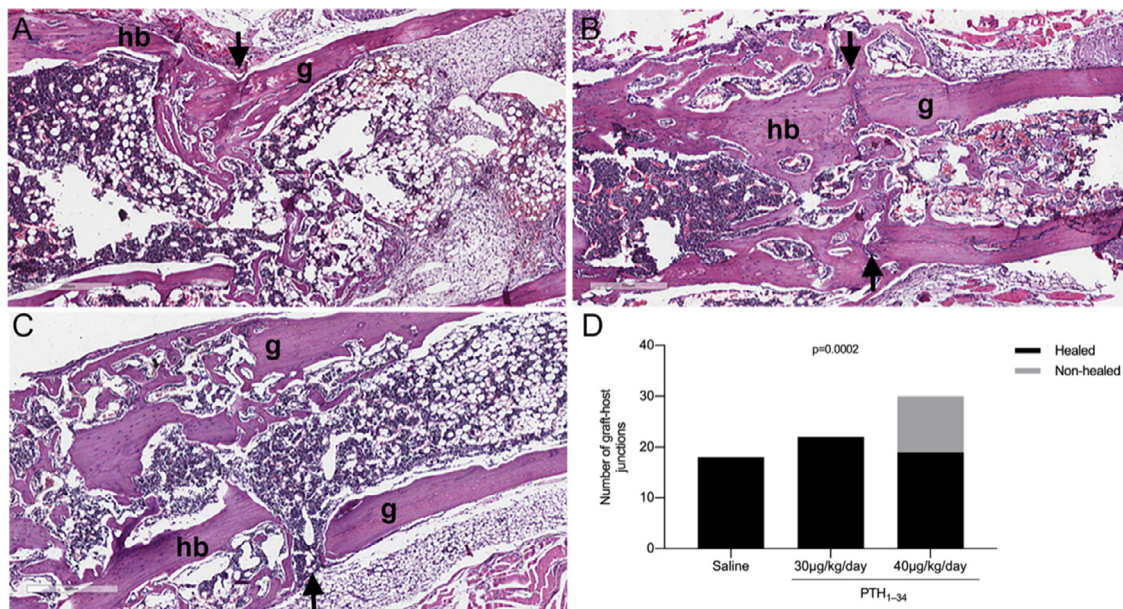


Fig. 6. Histological assessment of graft-host osseointegration at 14 days postsurgery. Graft-host integration was determined when new bone formation originating from the endosteal and/or periosteal surfaces provided the union between the graft (g) and the host bone (hb) junctions. (A) Representative sagittal section of grafted femur in untreated control animal showing graft-host union (arrow). (B) In 30 µg/kg/day PTH₁₋₃₄ allograft healing (arrows) and incorporation is enhanced compared to 40 µg/kg/day PTH₁₋₃₄ whilst it was similar to untreated controls. (C) In 40 µg/kg/day PTH₁₋₃₄ discontinuities at the graft-host junctions (arrow) were observed in 11 out of 30 interfaces. (D) Bar plots represent healed and non-healed graft-host junctions. H&E staining. Scale bar: 400 µm (A and C); 300 µm (B).

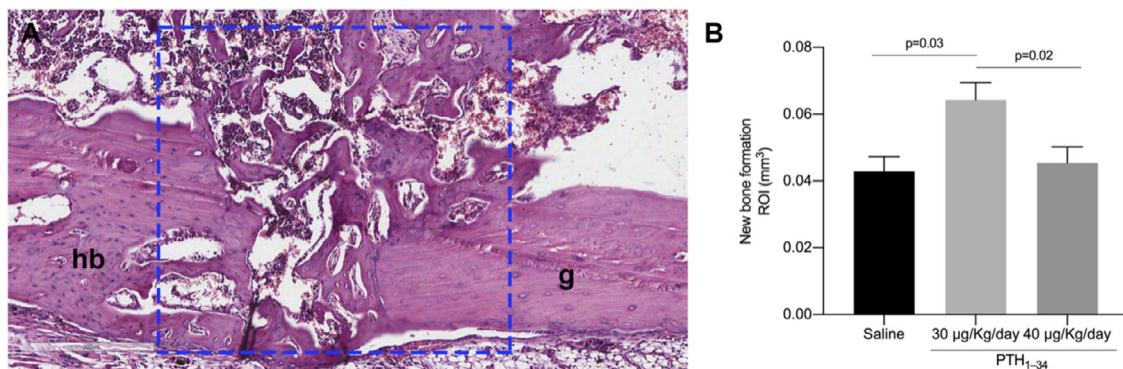


Fig. 7. Quantitative assessment of allograft integration with the host bone. (A) Micrograph showing representative sagittal section of graft-host junction in the region of interest ROI (dotted blue square) where bone healing quantification was performed. (B) In animals treated with intermittent 30 µg/kg/day PTH₁₋₃₄ bone formation at the graft-host junction was significantly greater than in 40 µg/kg/day PTH₁₋₃₄ as well as in untreated controls. H&E staining. g=graft, hb=host bone. Scale bar: 300 µm. Bar plots represent mean ± SEM.

In the clinical scenario, graft revitalization is necessarily associated with the successful treatment of segmental defects with allogeneic grafting. In our current murine model, the intermittent daily treatment with 30 µg/kg PTH₁₋₃₄ initiated immediately after surgery and maintained continuously for 14 days supported increased osteogenesis that resulted in the biologically superior revitalization of the devitalized graft compared to 40 µg/kg/day PTH₁₋₃₄. At the lower dose, the bone tissue was restored throughout the allograft marrow cavity and promoted continuous host-graft integration by means of interconnected cancellous bone trabeculae in close association with hematopoietic cells and bone marrow sinusoids. Moreover, the volume of bone formed at the graft-host junction, in addition to being greater than the volume formed from the 40 µg/kg/day PTH₁₋₃₄ dose regimen, overpassed the contact zone with the host bone and extended to both endosteal and periosteal surfaces.

It is noteworthy that there is no consensus in the literature on the optimal PTH₁₋₃₄ dosing regimen that is most effective at increasing bone formation and consolidation of bone grafts in

murine models. Doses ranging from 2.5 to 200 µg/kg/day have been used to investigate the anabolic effect of PTH₁₋₃₄ used to treat clinically less demanding orthopedic scenarios, such as fractures and bone loss due to osteopenia/osteoporosis [42,44–47]. In a more challenging scenario like structural allograft healing, previous reports [21,39,40] demonstrated that intermittent PTH at a standard dose of 40 µg/kg/day produced an anabolic effect on bone and enhanced allograft osseointegration and biomechanics. Although the focus of the present study was not to address the issue of the optimal dose of PTH₁₋₃₄ to promote the incorporation of allogeneic allograft, we believe our findings on structural allograft healing add relevant information that demonstrates that intermittent PTH₁₋₃₄ treatment at a lower dose could eventually be considered as an option to replace the standard dose of 40 µg/kg/day in future translational models in rodents. Another issue that remains unclear is the choice of the best therapeutic window for treatment with PTH₁₋₃₄. Depending on when treatment is started, different cell populations that respond to the drug are activated, determining on a priority

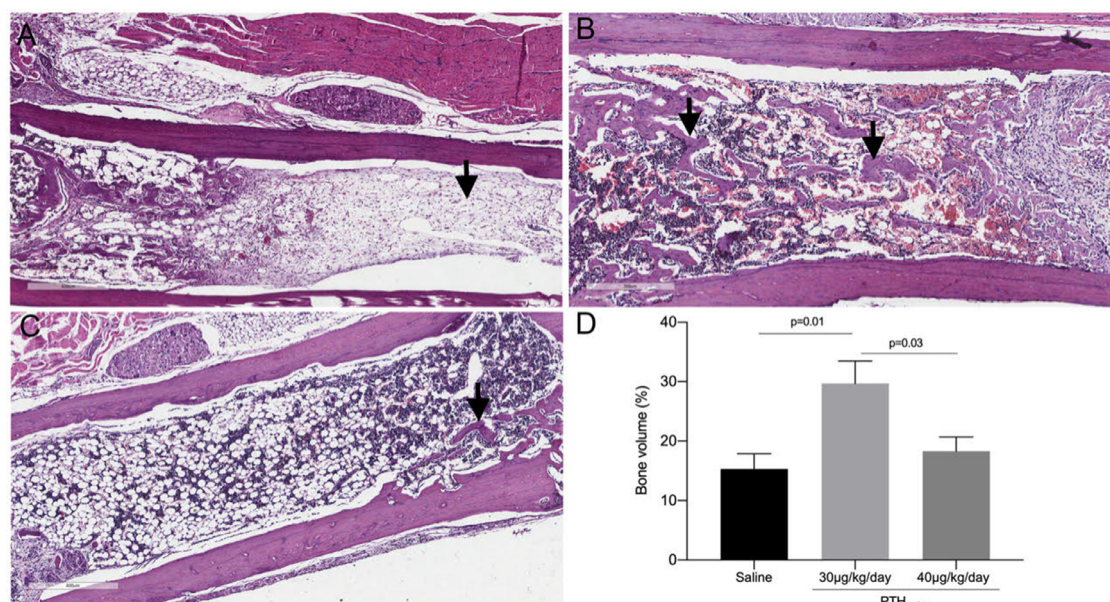


Fig. 8. Histomorphometric assessment of graft revitalization expressed as the percentage of newly formed mineralized bone in the marrow cavity of the graft. (A) Representative sagittal section of grafted femur in untreated control animal showing the inner portion of the graft mostly filled with loose vascularized connective tissue lacking evidence of osteogenic activity (arrow). (B) Intermittent daily treatment with 30 µg/kg PTH₁₋₃₄ for two weeks resulted in significantly increased revitalization of allogeneic grafts. Note that the allograft filling reproduced the sponge-like aspect of cancellous bone and also the microenvironment of the medullary cavity, which includes the repopulation with hematopoietic bone marrow (arrows). (C) In 40 µg/kg/day PTH₁₋₃₄ new bone formation is scanty (arrow) and resulted in reduced bone graft revitalization that was insufficient to promote continuous host-graft integration. Scale bar: 400 µm (A and C); 300 µm (B). Bar plots represent mean ± SEM.

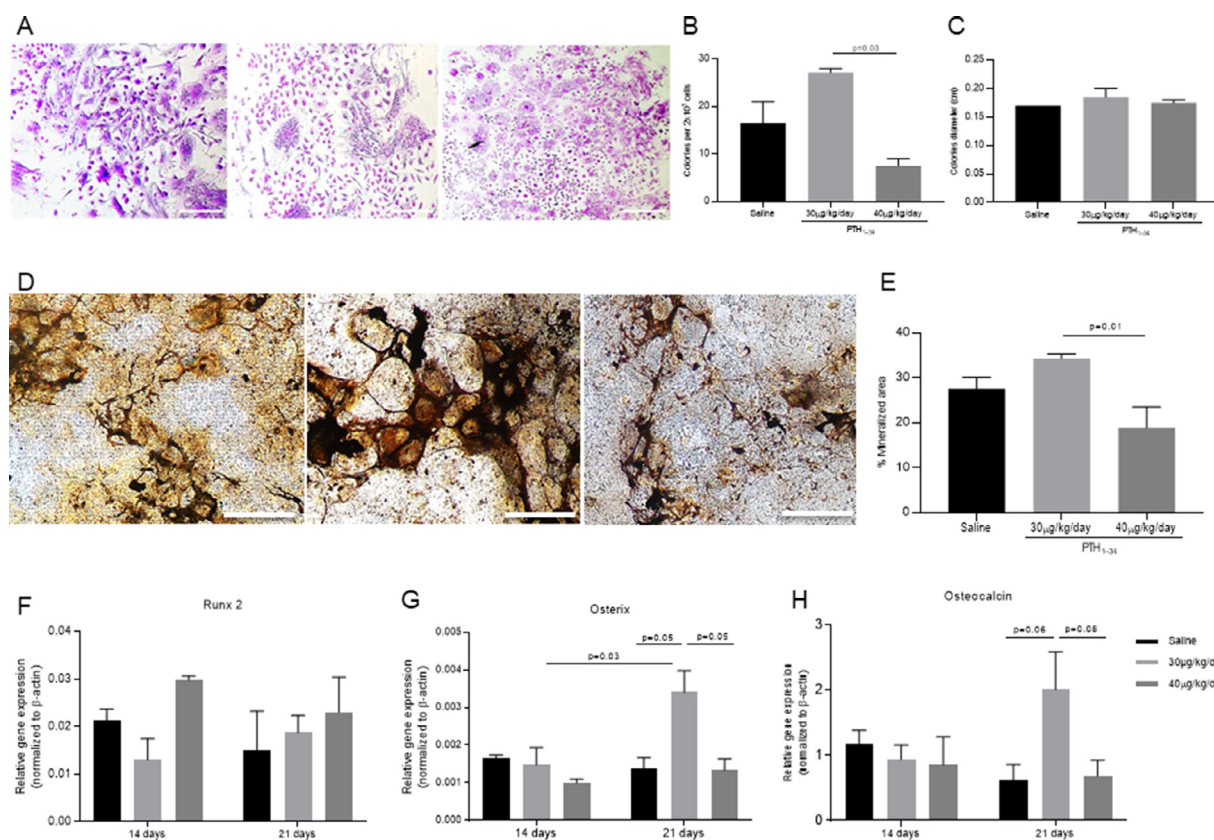


Fig. 9. Frequency and osteogenic capacity of BMSC after *in vivo* PTH₁₋₃₄ administration to C57BL/6 mice for 7 days. (A) Established clonal cultures of BMSCs. After 14 days, colonies with greater than 50 cells were counted and measured within each group. (B) The total number of colonies was significantly more numerous in 30 µg/kg/day PTH₁₋₃₄ compared to 40 µg/kg/day PTH₁₋₃₄. (C) Measurement of mean colony diameter in each group (n/s, not significant). (D) The gross appearances of the culture dishes stained by Von Kossa method. (E) Mineral nodules as a measure of the osteogenic capacity of osteoprogenitor cells were increased post *in vivo* treatment with 30 µg/kg/day PTH₁₋₃₄ compared to 40 µg/kg/day PTH₁₋₃₄ and control samples. (F) Intermittent PTH₁₋₃₄ treatment had no significant effects on *Runx2* expression. (G) In 30 µg/kg/day PTH₁₋₃₄ the expression of osteogenic differentiation-specific gene *Osterix* increased over time and was upregulated in comparison to both the 40 µg/kg/day PTH₁₋₃₄ and the saline groups. (H) Mineralization-specific gene *Osteocalcin* was overexpressed on 30 µg/kg/day PTH₁₋₃₄ compared to 40 µg/kg/day PTH₁₋₃₄ and saline groups though it failed to reach statistical significance. For each experiment, data are averaged in triplicate from two mice and expressed as mean ± SD. Scale bar: 300 µm (A) and 100 µm (D).

scale the intensity of events related to the different stages of the bone repair process.

To reproduce an experimental model aiming to investigate therapeutic measures to enhance the incorporation of allografts, it is desirable that throughout the bone repair process the graft be maintained in conditions of biomechanical stability [48]. Furthermore, the situation of a critical size defect needs to be corroborated by the absence of callus formation and spontaneous consolidation in defects that are left empty (not reconstituted) [49,50]. As in the clinical situation, osteosynthesis with micro locking plate provides adequate stabilization for the reconstruction of segmental bone defects in mice [51].

In alignment with these premises, our choice for stabilization with plate and screws enabled us to assess the rate of graft revitalization including the establishment of continuous host-graft integration and restoration of the bone marrow microanatomy. In addition, stable fixation guaranteed uniform biomechanical conditions for consolidation to occur, eliminating variables such as the formation of endochondral callus and maintained the graft positioned in the defect during the experimental period. In our opinion, an additional suture cerclage encompassing the plate and the graft helped to keep the grafts anatomically positioned at the defect site. Finally, this type of stabilization allowed the animals to be subjected to daily manipulation for two weeks without causing loosening of the screws or displacement of the grafts.

Regarding the rates of allograft revitalization and consolidation, our results cannot be compared with that of other authors, due to differences between stable fixation and fixation with intramedullary nail [2,39,40,47,52]. As far as we know, there are no reports in the literature on the role of PTH₁₋₃₄ in the reconstruction of critical bone defects in mice with structural grafts stabilized with a plate and blocked screws.

Our *in vivo* findings were confirmed by the *in vitro* experiments that showed that 30 µg/kg/day PTH₁₋₃₄ increased the frequency and the osteogenic capacity of osteoprogenitor cells in the bone marrow. Previous *in vitro* studies have demonstrated that osteogenic differentiation induced by PTH on BMSC is not a dose-dependent event, but rather higher doses seem to mitigate osteogenic differentiation of precursor cells [53]. In a non-surgical animal model in senescent osteopenic mice, Saidak et al. [54] showed that intermittent treatment with PTH at a lower dose was effective at increasing bone formation and axial bone volume through enhanced osteoblast activity and modulation.

Some genes directly related to bone formation such as *Osterix* and *Runx2*, activated during the exposure of BMSCs to PTH₁₋₃₄, are involved in the consolidation of fractures in animal models [47]. Our studies showed that PTH₁₋₃₄ was effective at increasing the expression of *Osterix* (osteoblast-specific transcription factor which activates during differentiation of preosteoblasts) and tended to increase the expression of *Osteocalcin* (the gene which encodes a bone protein secreted by osteoblasts) after *in vitro* induction of osteogenic differentiation of BMSC from mice previously treated with 30 µg/kg/day PTH₁₋₃₄. The expression level of *Osterix* displayed a time-dependent increase pattern and was upregulated compared to the higher PTH dosing regimen and saline-injected controls, indicating that the lower PTH₁₋₃₄ dose accelerates osteoinduction of BMSCs. These results are corroborated by previous reports that demonstrate that intermittent treatment with PTH₁₋₃₄ enhances *Osterix* expression and promotes BMSC differentiation into mature osteoblasts [47]. Intermittent PTH₁₋₃₄ treatment had no significant effects on *Runx2* expression. This finding is supported by previous reports showing that *Runx2* is involved in the initiation of differentiation of osteoprogenitor cells into osteoblasts. High levels of gene expression are followed by a progressive reduction in the later stages of cell differentiation, as low *Runx2* expression is required for the maintenance of mature osteoblast function [55]. Fur-

ther studies assessing the earlier stages of BMSC differentiation are necessary to delineate more precisely the molecular mechanisms of PTH induction on *Runx2* expression.

One of the strengths of this study was the use of a highly reproducible model of a critical segmental defect reconstructed with allogeneic structural grafts, stabilized with a plate and blocked screws. The osteosynthesis material permitted the positioning of the plate with precision, allowing for the creation of defects of the same size and for the analysis of the extension and quality of the graft fill tissue. Another positive point of this type of implant was the stability of the fixation. If we consider that the formation of the endochondral callus is an indirect way of evaluating interfracture movement, the absence of cartilage in the samples that were analyzed confirms that this type of synthesis reproduces a stable fixation. A limitation that was identified was the lack of proof of the quality of the consolidation by biomechanical tests and the evaluation of the action of PTH₁₋₃₄ on the incorporation of the grafts in a single observation point.

Conclusion

Our results suggest that intermittent PTH₁₋₃₄ treatment at 30 µg/kg/day after femoral allograft reconstruction surgery accelerated the healing process as evidenced by new bone formation induced on endosteal and periosteal surfaces, enhanced revitalization of the allogeneic graft, and increased frequency and osteogenic capacity of osteoprogenitor cells from the bone marrow. These findings should encourage further studies aimed at investigating the potential therapeutic use of intermittent PTH, in particular with regards to the optimal dosing regimen in clinically challenging orthopedic scenarios.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.injury.2021.03.063](https://doi.org/10.1016/j.injury.2021.03.063).

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