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# The local administration of parathyroid hormone encourages the healing of bone defects in the rat calvaria: Micro-computed tomography, histological and histomorphometric evaluation

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ABSTRACT

*Objective:* To evaluate the effect of a single-dose local administration of PTH on bone healing in rat calvarial bone defects by means of micro-computed tomography, histological and histomorphometric analysis.
*Design:* Critical-size cranial osteotomy defects were created in 42 male rats. The animals were randomly divided into 3 groups. In the C Group, the bone defect was only filled with a blood clot. In the S Group, it was filled with a collagen sponge and covered with bovine cortical membrane. In the PTH Group, the defect was filled with a collagen sponge soaked with PTH and covered with bovine cortical membrane. The groups were further split in two for euthanasia 15 and 60 days post-surgery. Data was statistically analyzed with *t*-tests for independent samples or the nonparametric Mann-Whitney test when applicable. Intragroup comparisons were analyzed with paired *t*-tests ($p < 0.05$).
*Results:* Micro-CT analysis results did not demonstrate statistically significant intergroup differences. At 15 days post-surgery, the histomorphometric analysis showed that the PTH Group exhibited a significantly higher percentage of bone formation compared with the S Group. At 60 days post-surgery, a higher percentage of new bone was observed in the PTH group.
*Conclusion:* The results suggest that the local administration of PTH encouraged the bone healing in critical-size calvarial defects in rats.



## 1. Introduction

Bone remodeling is the continuous removal of bone followed by the synthesis of bone matrix and mineralization. This process is mediated by calcium homeostasis, which needs three hormones for balance: PTH, Vitamin D (calcitriol) and calcitonin (Aggarwal and Zavras, 2012; Li et al., 2013; Tokunaga et al., 2011).

Parathyroid hormone (PTH) is composed of 84 amino-acid proteins. PTH exhibits both direct and indirect effects, particularly in bone remodeling, kidney and intestines, acts constantly to maintain the optimal endogenous concentration of calcium ions in the bloodstream and is also used in the treatment of osteoporosis (Aggarwal and Zavras, 2012; Alkhiary et al., 2005; Andreassen, Ejersted, & Oxlund, 1999; Li et al., 2013; Mair et al., 2009; Skripitz, Andreassen, & Aspenberg, 2000; Tokunaga et al., 2011; Yun et al., 2010).

Parathyroid hormone exerts both anabolic and catabolic action on the bone (Aggarwal and Zavras, 2012; Andreassen et al., 1999; Kempen et al., 2010; Li et al., 2013; Mair et al., 2009; Ohkawa, Tokunaga, & Endo, 2008; Pensak et al., 2015; Skripitz et al., 2000; Skripitz, Johansson, Ulrich, Werner, & Aspenberg, 2009; Tokunaga et al., 2011; Yun et al., 2010). Preclinical studies have shown that the use of PTH for the treatment of osteoporosis improves bone fracture healing (Alkhiary et al., 2005; Andreassen et al., 1999; Neer et al., 2001). Its intermittent administration increases bone remodeling with greater effect on bone apposition, thereby leading to increased microarchitecture and bone volume rather

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than bone resorption (Gabet, Kohavi, Muller, Chorev, & Bab, 2005; Kneissel, Boyde, & Gasser, 2001). It plays an important role in bone turnover and calcium homeostasis, renal excretion of inorganic phosphate and activation of vitamin D (Aggarwal and Zavras, 2012; Li et al., 2013; Mair et al., 2009; Skripitz et al., 2000; Tokunaga et al., 2011; Yun et al., 2010).

PTH triggers bone resorption due to its effects on osteoblast activity, as PTH receptors are found on osteoblast membranes. PTH binds to PTH1R (parathyroid hormone 1 receptor) on osteoblasts to stimulate the secretion of RANKL (receptor activator of nuclear factor-kB ligand). RANKL binds to receptor activator of RANK on the surface of osteoclasts and promotes cell differentiation and maturation of immature osteoclasts, thus prolonging their survival. PTH also decreases the osteoblasts' secretion of osteoprotegerin. Osteoprotegerin is a protein secreted by osteoblasts that effectively binds to RANKL and prevents its attachment to RANK, thus decreasing the differentiation and resultant maturation of osteoclasts and bone resorption. While PTH and glucocorticoids decrease the production of osteoprotegerin, estrogen increases its expression (Simonet et al., 1997; Yun et al., 2010).

Previous studies have evaluated the effects of systemic PTH administration in rats on bone healing of calvarial critical-size defects (Andreassen and Cacciafesta, 2004; Silva et al., 2015; Stancoven et al., 2013; Tsunori et al., 2015; Tsunori, 2015; Yun et al., 2010), post-extraction socket regeneration (Kuroshima, Al-Salihi, & Yamashita, 2013), bone response around implants in osteoporotic rat maxillae (Heo, Park, Jeon, & Pyo, 2016; Park, Heo, Kim, Min, & Pyo, 2016), periodontal defects repair (Wang, Du, & Ge, 2016) and periodontitis (Barros, Silva, Somerman, & Nociti, 2003). To the best of our knowledge, only one study to date has evaluated the effects of the local administration of PTH in rat gingiva on alveolar bone regeneration (Tokunaga et al., 2011). Thus, the local effects of this drug should be further studied and understood.

The aim of this study was to evaluate the effect of a single-dose local administration of PTH on bone healing in rat calvarial bone defects by means of micro-computed tomography as well as histological and histomorphometric analysis.

## 2. Material & methods

### 2.1. Experimental design

Ethical approval was obtained from the Ethics Research Committee of Positivo University. The experiment was carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Forty-two male rats (Rattus norvegicus, Albinus, Wistar) aged 3–5 months and weighing an average of 387 g were used. The animals had ad libitum access to water and a standard laboratory diet.

Throughout the trial period, the animals were triple-housed in cages in purpose-designed rooms. The environmental conditions of light, temperature and humidity were controlled. The animals were randomly divided into three groups: C Group (control), S Group (collagen sponge) and PTH Group (parathyroid hormone). The groups were further split in two (n = 7) for euthanasia 15 and 60 days post-surgery.

### 2.2. Surgical procedure and PTH administration

For the experimental surgical procedures, the animals were sedated with oxygen and isoflurane 3 l/min (Cristália, Itapira, SP, Brazil) and then anesthetized by intramuscular injection in the back of the thigh with Xylazine 10 mg/kg (Vetbrands, Paulinia, SP, Brazil) and ketamine 80 mg/kg (Vetbrands). Anesthesia was maintained by isoflurane vaporization as needed. The surgical procedure was performed by one single trained and calibrated operator.

After the induction of anesthesia, the dorsal part of the cranium was shaved and aseptically prepared for surgery. A U-shaped incision was performed with a scalpel blade No. 15c (Advantive, Xishan City, China) for surgical access to the calvaria area and a full-thickness flap was raised in the posterior direction.

A critical-size (5 mm diameter), through-through, cranial osteotomy defect was created using a trephine bur (Neodent, Curitiba, Brazil) engaged in an implant hand piece (20:1, Kavo, Joinville, Brazil) under profuse irrigation with sterile saline. A defect was created on the skull of each animal for a total of 42 bone defects. The defect was centered along the median sagittal suture 1 mm anterior to the occipital bone. The resulting calvarial block was then carefully removed to avoid tearing the dura.

In the C Group, the bone defect was only filled with a blood clot. In the S Group, it was filled with a 5 mm in diameter absorbable hemostatic collagen sponge (Hemospon, Technew, Rio de Janeiro, RJ, Brazil) that was cut with the trephine bur so that its size was compatible with the defect, and then covered with bovine cortical membrane (GenDerm, Baumer, São Paulo, SP, Brazil) cut to fully cover the sponge and defect (size 8 × 8 mm). In the PTH Group, the defect was filled in a manner similar to the S Group. However, the sponge was soaked with 20 μg PTH (Forteo, Indianapolis, Indiana, United States). Finally, tissues were repositioned and sutured using 4-0 silk (Ethicon Inc. Somerville, NJ, USA).

The animals received a single 0.1 ml intramuscular dose of antibiotic (Pentabiotic 24,000 IU/kg body wt) and 0.1 ml subcutaneous morphine immediately post-surgery. For 3 days, they were given paracetamol dissolved in water (20 drops/400 ml).

The animals were euthanized at either 15 or 60 days post-surgery using a $CO_2$ chamber for 5–10 min. The original area of surgical defects in the calvaria were then harvested and fixed in 10% buffered formalin solution for 24 h, after soft tissue has been removed.

### 2.3. Micro-computed tomography analysis

Micro-computed tomography (micro-CT) scans containing the surrounding defects of all samples were obtained 60 days post-surgery using a micro-CT apparatus (Skyscan, 1174 v.2, Kontich, Belgium).

The micro-CT was set as follows: 0.5 mm aluminum filter; 1° rotation steps and an isotropic voxel size of 19.7 μm; voltage, 50 kV; and electrical current, 800 μA.

Three-dimensional images were produced using NRecon 1.66 computer software (Skyscan). The computer software DataViewer (Skyscan) was then utilized for the evaluation of three-dimensional reconstructions (linear measurements) of the coronal, transaxial and sagittal axes as well as the linear definition of the original bone defect in the anterior-posterior direction, lateral from the right parietal to the left parietal and linear extension of the original bone defect.

For volumetric analysis (tissue volume (TV), bone volume (BV) or percentage of bone volume (BV/TV)), an axis of interest (transaxial) was chosen using the CTAn 1.10 software (Skyscan) to measure the newly formed bone volume. To analyze the bone defects, only the 5 mm elliptical shape was chosen to select the area of interest.

### 2.4. Tissue preparation

The blocks used for histological and histomorphometric analysis were submitted to histotechnical preparation. The

sections were stained with hematoxylin and eosin (HE) for analysis using light microscopy.

### 2.5. Histological analysis

One single calibrated operator performed the histological analysis on three slides stained with HE of each animal.

The assessed parameters were the closing of the bone defect, connective tissue characteristics, the presence of osteoid matrix, the presence of chronic or acute inflammatory infiltrate, the progression of the type of healing present in the bone defect and thickness of newly formed tissues in relation to the original bone.

### 2.6. Histomorphometric analysis

For the histomorphometric analysis, all slides were photographed covering both defect edges. Using the ImageJ® 1.6.0 software (Wayne Rasband (NIH), Bethesda, MA, USA) the following histomorphometric measurements were performed: 1) Total area (TA): measurement of the total area of the surgically created bone defect; 2) Newly formed bone area (NBA): measurement of the newly formed bone area; 3) Soft tissue area within the bone defect (STA): measurement obtained by subtracting NBA from TA (STA = TA-NBA). TA, NBA and STA were measured in $mm^2$ and TA was considered 100% of the area to be analyzed.

### 2.7. Statistical analysis

Data was expressed as means and standard. The Student *t*-test for independent samples or, when applicable, the nonparametric Mann-Whitney test was performed to compare the differences between groups. Intragroup comparisons were analyzed with paired *t*-tests. Results were regarded as significant at $p < 0.05$. All analyses were performed using the statistical software Statistical Package for Social Science (SPSS; version 20.0; SPSS Inc. Chicago, IL, USA).

## 3. Results

### 3.1. Concentration of serum calcium

The intragroup comparison showed that in the C Group, at 60 days post-surgery, the final concentration of serum calcium was significantly lower than the initial concentration ($p = 0.005$). The other groups did not show statistically significant changes with respect to this parameter. The PTH Group demonstrated an increase in serum calcium concentration 60 days post-surgery but without statistical significance (Table 1).

### 3.2. Micro-CT analysis

Micro-CT analysis results did not demonstrate statistically significant intergroup differences with respect to tissue volume (TV), bone volume (BV) or percentage of bone volume (BV/TV) 60 days post-surgery (Table 2 and Fig. 1). The PTH Group showed a higher percentage of new bone formation compared to the other two groups but without statistically significant difference.

**Table 1**
Concentration of serum calcium (mg/dL) at different stages (n = 4).

| Group | Days | Initial Mean (SD) | Final Mean (SD) | p value |
|---|---|---|---|---|
| C Group | 15 | 9.90 (0.35) | 9.67 (0.13) | 0.336 |
| | 60 | 10.06 (0.25) | 9.19 (0.07) | 0.005* |
| PTH Group | 15 | 11.66 (0.66) | 10.32 (0.76) | 0.147 |
| | 60 | 10.25 (0.56) | 11.16 (0.52) | 0.081 |

SD: standard deviation. C Group (control), PTH Group (parathyroid hormone).
* Statistically significant at $P < 0.05$, paired *t*-test.

**Table 2**
Micro-CT analysis results of percentage of newly formed bone within the bone defect area 60 days post-surgery (n = 7).

| Variable | C Group Mean (SD) | S Group Mean (SD) | PTH Group Mean (SD) |
|---|---|---|---|
| Tissue volume (TV) | 1.30 (0.42) | 1.15 (0.00) | 1.31 (0.42) |
| Bone volume (BV) | 0.12 (0.10) | 0.08 (0.06) | 0.17 (0.17) |
| Percentage of bone volume (BV/TV) | 9.42 (9.70) | 6.94 (5.23) | 12.30 (11.32) |

SD: standard deviation. P values showed no statistical difference, *t*-test ($P > 0.05$). C Group (control), S Group (collagen sponge), PTH Group (parathyroid hormone).

### 3.3. Histological analysis

In most of the C Group samples, the defect sites were filled with collagen fibers and with new bone formation near the edges of the defect at 15 and 60 days post-surgery. In the S Group, they were filled with collagen fibers, chronic inflammatory processes and membrane remains, and with new bone formation near the edges of the defect, in both times. In the PTH Group, the defect sites were filled with dense connective tissue, chronic inflammatory processes (more evident at 15 days post-surgery) and new bone formation permeating the connective tissue and near the edges of the defect, in both times (Figs. 2 and 3).

### 3.4. Histomorphometric analysis

At 15 days post-surgery, the PTH Group exhibited a significantly higher percentage of new bone formation (NBA) than the S Group ($p = 0.003$). The same was observed when the C Group was compared to the S Group ($p = 0.007$) (Table 3).

The percentage of soft tissue area (STA) within the bone defect area in the S Group was significantly higher than the PTH ($p = 0.007$) and the C ($p = 0.012$) Groups at 15 days post-surgery (Table 4).

## 4. Discussion

Current studies of the literature reported that intermittent systemic administration of PTH enhanced bone healing in calvarial defects in rats (Tsunori et al., 2015; Tsunori, 2015; Yun et al., 2010), on implants inserted in the tibia (Li et al., 2013; Mair et al., 2009; Skripitz et al., 2009) and on periodontal defects (Kuroshima et al., 2013). However, the effect of local administration of PTH is still unclear. The aim of this study was to evaluate the effect of a single-dose local administration of PTH on bone healing in rat calvarial bone defects. The purpose of this methodology was to simulate a clinical situation, since patients may not accept the intermittent systemic PTH therapy with the purpose of stimulating intraoral bone formation.

Tokunaga et al. (Tokunaga et al., 2011) reported the effect of local administration of PTH on alveolar bone recovery with intermittent applications in rat gingiva after inducing experimental periodontitis. Results suggested that the topical and intermittent administration of PTH can increase the alveolar bone mass and may be effective for periodontal tissue regeneration.

In the present study, PTH was also administered locally, but in a single dose, soaked in an absorbable collagen sponge associated with a bovine cortical membrane. Results showed a greater amount of newly formed bone in the PTH Group 15 days post-surgery compared with the S Group (Table 3), suggesting that PTH acted locally to stimulate the initial bone healing. At 60 days post-surgery, there was no statistically significant difference between

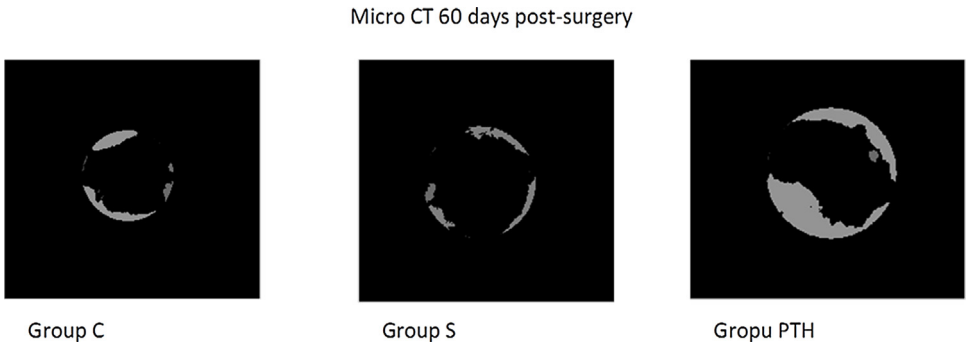


**Fig. 1.** Images of new bone formation in groups C, S and PTH, by computerized microtomography analysis at 60 days postoperative. PTH group with greater bone neoformation compared to the other groups. Group C with intermediate bone neoformation and Group S with least bone neoformation.

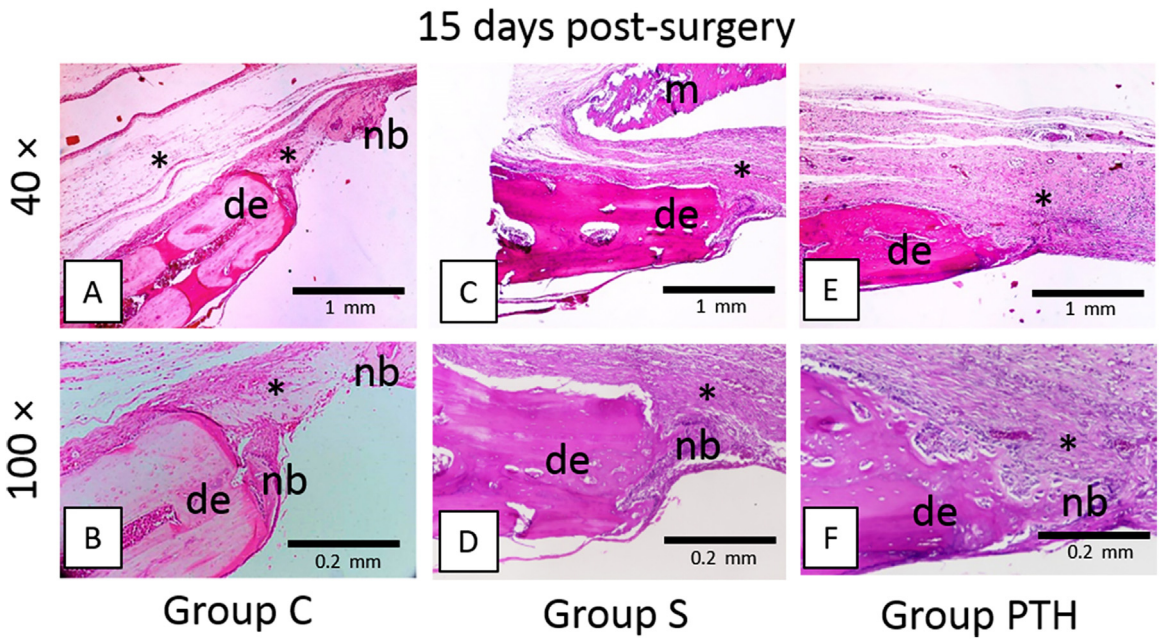


**Fig. 2.** Histological sections of critical size defects in 15 days post-surgery for all groups (H.E. staining). **Group C:** New bone formation is observed on the edge of the defect and the predominant healing is characterized by fibers of connective tissue. **Group S:** Fibers of connective tissue with chronic inflammatory processes, membrane remains and new bone formation near the edges of the defect were observed. **Group PTH:** New bone formation is observed close to the edge of the defect, and more to the center the predominant healing is characterized by fibers of connective tissue. * = connective tissue. **de** = defect edge. **nb** = new bone. **m** = membrane.

groups although on average, there was a greater amount of bone formation in the PTH Group than in the other groups; this is compatible with the idea that PTH has a positive effect on bone healing in the calvaria of rats.

The method of creating critical-size defects in rat calvaria was chosen since it is well established and widely used in the literature. Recently, Tsunori et al. (Tsunori et al., 2015) performed a study similar to our experiment, but using systemic administrations of the hormone. Each of the 10 rats was treated with 35 or 105 mg/kg PTH by intraperitoneal injection 3 times per week for twelve weeks. The control group was injected with sterile saline instead of PTH. Results indicated that the greater the dose of intermittent PTH, the greater the bone formation beyond the skeletal envelope in the rat calvaria. The authors suggested that a lower frequency of PTH administration with a higher dose could be a possible protocol design for bone regeneration therapy. However, there is still no consensus in the literature with respect to the ideal dose of PTH to be systemically administered with the purpose of enhancing bone formation. Different doses and administration intervals have demonstrated successful results (Kuroshima et al., 2013; Li et al., 2013; Mair et al., 2009; Skripitz et al., 2000; Tsunori et al., 2015; Tsunori, 2015; Yun et al., 2010).

Since the local application of PTH is a more recent therapy option for bone gain, a protocol for how it should be administered and the dose required have not been established. Therefore, we can state that this is a pioneer study, which makes it difficult to compare the results obtained. Promising results of bone healing were observed when 20 μg of PTH (a single dose of Fortéo®) was locally administered during surgery. The dose used in the present study was lower than the one used in the study conducted by Tsunori et al. (Tsunori et al., 2015). Still, new bone formation was observed. Further studies are needed to assess the dose necessary to achieve bone healing with this therapy.

When systemically administered, PTH acts by stimulating calcium release into the bloodstream (this release occurs through an initial bone resorption) and thereby activating vitamin D, which then helps the circulating calcium to form new bone. Yet, in the mechanism of action of calcium homeostasis, it is important to mention the role of the PTH receptors on osteoblasts, called PTH1R. If the receptor is active in osteoblasts, initial bone resorption takes

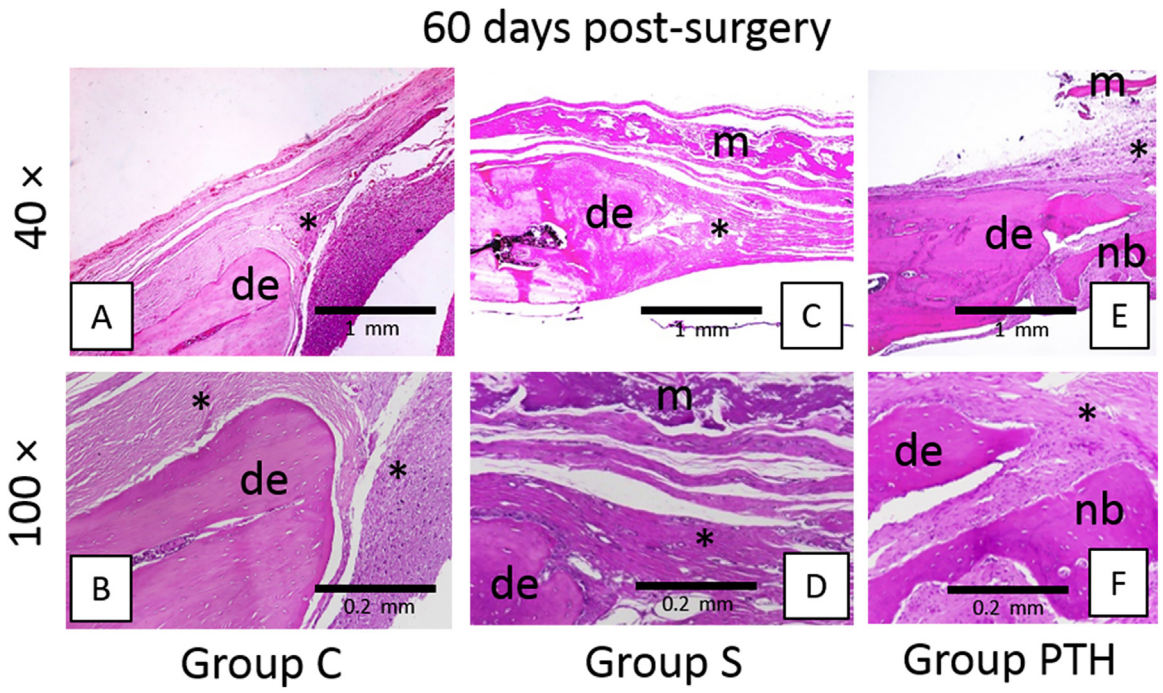


**Fig. 3.** Histological sections of critical size defects in 60 days post-surgery for all groups (H.E. staining). **Group C:** Similarly to 15 days post-surgery, healing was characterized by a fibrous connective tissue. **Group S:** A fibrous connective tissue was observed. The membrane was still present, similarly to 15 days post-surgery. **Group PTH:** Pattern of histological healing similar to the 15 days post-surgery. More bone formation was observed near the edges of the defect surrounded by a fibrous connective tissue. * = connective tissue. **de** = defect edge. **nb** = new bone. **m** = membrane.

**Table 3**
Histomorphometric analysis results of percentage of newly formed bone area (NBA) within the total area (TA) of the surgically created bone defect and comparison intergroups (n = 7).

| Days | C Group | S Group | PTH Group | C Group vs. S Group p value[b] | PTH Group vs. S Group p value[b] | PTH Group vs. C Group p value[a] |
|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Mean (SD) | | | |
| 15 | 16.2 (10.8) | 2 (3.8) | 12.8 (6.6) | 0.007* | 0.003* | 0.535 |
| 60 | 17 (17) | 20 (8) | 32.2 (14.4) | 0.716 | 0.063 | 0.09 |

SD: standard deviation. C Group (control), S Group (collagen sponge), PTH Group (parathyroid hormone).
[a] *t*-test.
[b] Mann–Whitney test.
* Statistically significant at $p < 0.05$.

**Table 4**
Histomorphometric analysis results of percentage of soft tissue area (STA) within the total area (TA) of the surgically created bone defect and comparison intergroups (n = 7).

| Days | C Group | S Group | PTH Group | C Group vs. S Group p value[b] | PTH Group vs. S Group p value[b] | PTH Group vs. C Group p value[a] |
|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Mean (SD) | | | |
| 15 | 84 (9.2) | 97 (4.4) | 86.8 (6.4) | 0.012* | 0.007* | 0.441 |
| 60 | 81 (20) | 80 (7.2) | 68.4 (14.4) | 0.917 | 0.062 | 0.275 |

SD: standard deviation. C Group (control), S Group (collagen sponge), PTH Group (parathyroid hormone).
[a] *t* test.
[b] Mann–Whitney test.
* Statistically significant at $p < 0.05$.

place, followed by bone formation. If the receptor is inactive, normal bone remodeling occurs (Martin and Ng, 1994; Simonet et al., 1997). Therefore, we may suggest that the receptor was active during the bone remodeling process in our study since a greater percentage of new bone formation was shown in the PTH Group 15 days post-surgery compared with the S Group. It would be necessary to perform an immunohistochemical analysis to detect the presence of PTH1R and verify if PTH actually had an anabolic effect and acted on the course of bone defect healing.

One of the analyses performed to check whether local PTH administration had systemic effects on the rats was measuring serum calcium concentration before the hormone administration and prior to euthanasia at 15 and/or 60 days post-surgery. The results demonstrated an increase in calcium concentration only in the PTH Group at 60 days post-surgery but without statistically significant differences within or between groups (borderline p value of 0.081, Table 1). This increase in serum calcium concentration suggests that the single-dose local administration

of PTH presents systemic catabolic effects. In the literature, only one study evaluated the intermittent systemic administration of PTH and assessed serum calcium concentration and alkaline phosphatase activity in rats (Tsunori et al., 2015). The authors also did not find a statistically significant difference in respect to calcium concentration changes between PTH and control groups.

When the Micro-CT imagens were analyzed, the PTH Group showed a higher percentage of new bone formation compared to the C Group and the S Group (Table 2). Even though this intergroup difference was not statistically significant, it corroborates with the results shown in the histological and histomorphometric analyses. In the study by Tsunori et al. (Tsunori et al., 2015), micro-CT analysis indicated a statistically significant difference between the control group and the PTH group with respect to the increase in bone volume after 8 weeks. Nevertheless, it is important to stress that in the study mentioned above, unlike ours, the effects of systemic administration of PTH were assessed.

Future studies using other types of carriers for local application or using intermittent local injections of the hormone, with different doses, associated or not to autogenous bone or a biomaterial, are needed to investigate if more complete bone regeneration can be achieved with local administration of PTH.

In conclusion, the results suggest that the local administration of PTH encouraged discreetly bone healing in critical-size calvarial defects in rats. Further research is needed to confirm the findings of this study.

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## Conflict of interest statement

The authors have nothing to disclose.

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