

Histomorphometry evaluation of bone anabolism promoted by prostaglandin E1 and its relation to hypercalcemia

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Abstract

Background: At present, parathyroid hormone is the only existing anabolic bone therapy but produces hypercalcemia. Prostaglandin E1 (PGE₁) has been suggested as a bone anabolic agent that allows bone modeling formation without producing hypercalcemia. This study aimed to corroborate these PGE₁ properties. **Methods:** For 22 days, rabbits ($n = 30$) were divided into three groups ($n = 10$ each group) and received intravenous solutions: vehicle (control group), palate disjunction + vehicle (sham group), and palate disjunction + 50 µg of PGE₁ (PGE₁ group). On days 1, 3, and 22, palatine suture X-rays were taken. On day 22, bone formation markers were analyzed, and the rabbits were sacrificed. Bone palate undecalcified samples were processed. Histomorphometry software was used to analyze bone parameters, and the mineralization front was stained with toluidine blue. Scalloped lines reflect remodeling-based bone formation (RBF), and smooth lines reflect modeling-based formation (MBF). **Results:** X-rays showed more significant palatal disjunction in the PGE₁ group; this group exhibited significant calcitriol serum increments. Hypercalciuria was observed in the PGE₁ group, and resorption markers (N-telopeptides) remained stable. Sutural bones in the PGE₁ group exhibited significant anabolism in structural parameters. RBF was 20%, and MBF was 6% in the sham group; in the PGE₁ group, RBF was 8.6%, and MBF was 17%. In the PGE₁ group, mineralization was significantly accelerated, but resorption remained stable. **Conclusions:** This model suggests that PGE₁ favors palate disjunction, calcitriol synthesis, and shortens the mineralization. Therefore, PGE₁ is an important bone anabolic molecule predominantly of modeling-based form and no hypercalcemia.

Keywords: Prostaglandin E1 bone anabolism. Calcitriol increments. Anabolism without hypercalcemia. Modeling-based bone formation. Remodeling-based bone formation and shortening bone mineralization.

Evaluación histomorfométrica del anabolismo óseo en la reparación promovida por la prostaglandina E1 y su relación con la hipercalcemia

Resumen

Introducción: La hormona paratiroides es la única molécula anabólica ósea, pero ocasiona hipercalcemia. La prostaglandina E1 (PGE₁) sugiere ser un anabólico óseo con formación por modelación predominante y generalmente no ocasiona hipercalcemia. El objetivo de este estudio fue corroborar estas propiedades de la PGE₁. **Métodos:** Por 22 días, 30 conejos divididos en tres grupos ($n = 10$ cada grupo) recibieron una solución por vía intravenosa: vehículo (grupo control), disyunción palatina más vehículo (grupo sham) y disyunción palatina más 50 µg de PGE₁ (grupo PGE₁). A los días 1, 3 y 22 se obtu-

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vieron radiografías de la sutura palatina. En el día 22 se analizaron los marcadores bioquímicos de formación ósea y se sacrificó a los conejos. Las suturas y los huesos suturales se procesaron sin descalcificar. La evaluación histomorfométrica fue digitalizada y el frente de mineralización ósea se tiñó con azul de toluidina. Las líneas irregulares reflejan resorción (remodelación) y las líneas rectas no resorción (modelación). **Resultados:** Radiográficamente, la disyunción palatina fue mayor en el grupo PGE₁. Este grupo mostró una hipercalcitonemia significativa, pero la calcemia y los marcadores resorptivos (N-telopeptídos) se mantuvieron estables. Por histomorfometría, los huesos suturales del grupo PGE₁ mostraron anabolismo significativo en parámetros estructurales. En el grupo sham, la remodelación ósea fue del 20% y la modelación fue del 6%; en el grupo PGE₁, la remodelación fue del 8.6% y la modelación fue del 17%. En este mismo grupo, la mineralización fue significativamente acelerada, pero la resorción se mantuvo igual. **Conclusiones:** Este modelo sugiere que la PGE₁ favorece la disyunción palatina y el aumento del calcitriol, y acelera la mineralización y el anabolismo óseo por modelación predominante sin hipercalcemia.

Palabras clave: Anabolismo óseo prostaglandina E1 (PGE1). Aumento del calcitriol. Anabolismo óseo sin hipercalcemia. Formación ósea por remodelación. Aceleración de la mineralización ósea.

Introduction

Prostanoids (PGs) are derived from fatty acids of cell membranes and released by phospholipase A2 (PLA₂)¹. PGs are classified into three series. Series 1 components are synthesized from homo- γ -linoleic acid, which is the origin of prostaglandin E₁ (PGE₁)².

For more than one decade, anabolic therapy for osteopenia has generated great enthusiasm. Four forms of the parathyroid hormone (PTH), 1-34, 1-84, 1-36, and abaloparatide, are currently the only anabolic bone therapy. PTH increases bone formation and bone resorption, causing hypercalcemia^{3,4}. An ideal anabolic bone treatment would optimize bone formation with minimal change in resorption and no hypercalcemia^{3,5}. Our objective was to investigate by biochemical and histomorphometry studies, including remodeling-based bone formation (RBF) and modeling bone formation (MBF), if PGE₁ might be a suitable bone anabolic agent that could be used in diseases such as osteoporosis, maxillary deficiency, and others.

Methods

This study was conducted following institutional, national, and international norms^{6,7}. Thirty 3-month-old New Zealand male rabbits of approximately 3 kg of body weight were divided randomly into three groups (n = 10): control group (with no palate disjunction + vehicle); sham group (with palate disjunction + vehicle), and PGE₁ group (with palate disjunction + prostaglandin administration). Rabbits were fed with a balanced diet (Ca, 1.2%; P, 0.8%; Vitamin D₃, 1 IU/g) and deionized distilled water *ad libitum*. Control and sham groups received 1 ml/day of vehicle (0.990 ml saline solution + 0.01 ml ethanol) intravenously for 22 days. The PGE₁

group received 50 μ g/ml of PGE₁ dissolved in 1 ml of vehicle solution intravenously for 22 days. The PGE₁ dose was calculated considering an 8% of lung inactivation⁸. Under pentobarbital sedation, basal X-rays from palatine suture were taken on days 1, 3, and 22 using radiologic plates at 0.08 mAmp/65 kV. The palatine suture was measured with a millimeter rack. After the basal X-ray, tensional palate disjunction was obtained with a metallic handle orthodontic fixer placed between the rabbits' incisive teeth in the sham and PGE₁ groups, with a pressure of 170 g/cm²². On day 6, the disjunction was fixed with a steel bar placed under the incisive teeth and a high curing resin, and the orthodontic handle fixer was retired. Since day 6, all rabbits received an intramuscular dose (40 mg) of oxytetracycline every 12 h/2 days. Ten days later, oxytetracycline administration was repeated.

On day 22, rabbits were sacrificed under deep general anesthesia. A last X-ray palatine suture and urine sample by bladder puncture were obtained to measure Ca, P, and cross-linked N-telopeptide^{9,10}. Blood samples from the aorta were also collected to measure total Ca and Mg (with atomic absorption flame spectrophotometry) and P levels (by quantitative phosphomolybdate complex spectrophotometry). Total alkaline phosphatase (AkPh) and its thermolabile bone fraction (AkPhO) were quantified by the modified Guttmann method and its bone isoform by the denaturation method⁹. Furthermore, PTH "intact" molecule (PtH₁), calcitonin (25 Vitamin D), and calcitriol (1,25(OH)₂ D₃) were quantified through radioimmunoassay. Samples of the anterior palatine bone were fixed in 70% alcohol solution and kept at room temperature. Subsequently, they were dehydrated by increasing ethanol concentrations and embedded undecalcified in methyl methacrylate. With a hard work microtome, 4 μ g thick sections

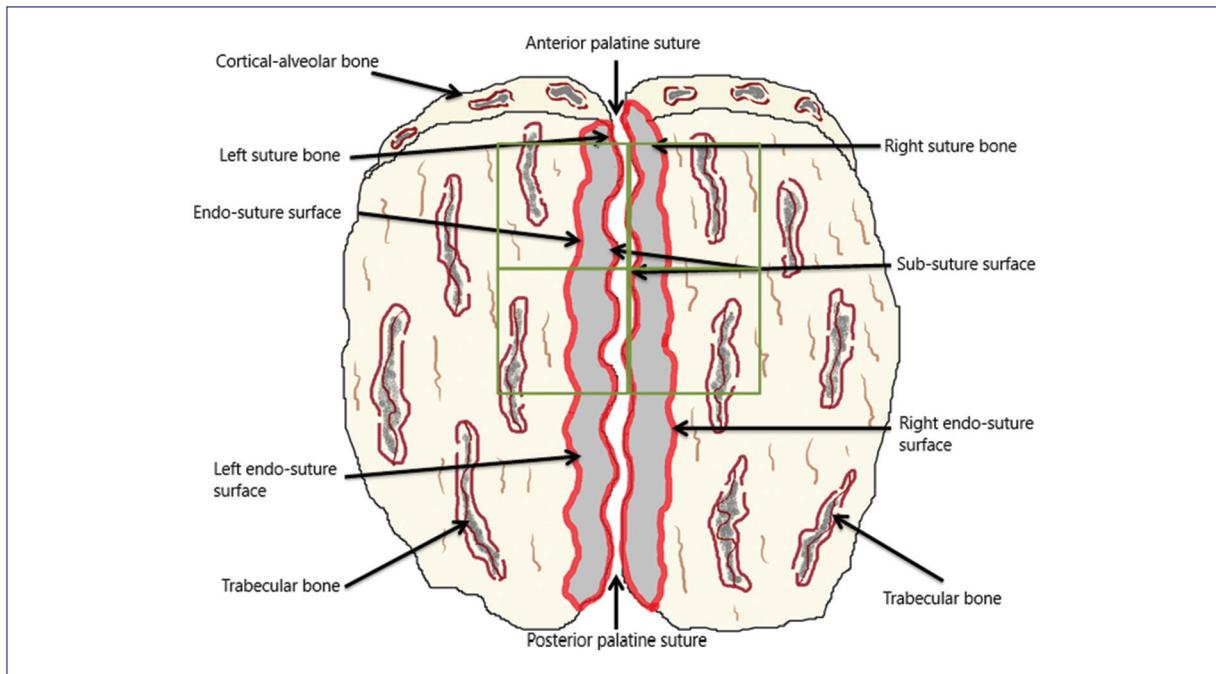


Figure 1. Anatomic diagram of the rabbit's palatine bone with the anterior and posterior regions. The square highlight of the anterior zone shows where the measures were performed; the anatomical identification of the bone surfaces is observed.

were obtained, rehydrated, and stained for Masson-Goldner trichrome, toluidine blue, and unstained for fluorescence microscopy. The suture bones (wormian) were measured, starting behind the cortical-alveolar bone towards the posterior region. The anterior, posterior, left, and right bone sutures surfaces and their sub-suture and endo-suture surfaces were identified (Fig. 1). The histomorphometry study of sutural bones was identified as structural, static bone formation, dynamic bone formation, and bone resorption. All analyses were performed using a digitizing table with an osteomeasure software (Osteometrics, Atlanta GA), following the nomenclature and recommendation of the American Society for Bone and Mineral Research¹¹⁻¹⁴.

The histomorphometry structural sutural-bone parameters (Sb): bone width (SBwi), bone area (SbAr), bone thickness (SbTh), and sutural bone volume (SbV/TV) were measured in the different groups.

The histomorphometry static bone formation parameters evaluated were osteoid thickness (SbOTh), osteoid surface/bone surface (SbOS/BS), osteoid volume/bone volume (SbOV/BV), and osteoblasts surface/BS (SbObS/BS). Other static parameters of bone formation were measured to analyze the sutural bone mineralization front (SbMF) in slides stained with toluidine blue, observing the architecture of the cement lines¹⁵. The

RBF was detected as cement lines scalloped due to prior osteoclast resorption. The MBF was identified as smooth cement lines as if they were forming on a quiescent BS¹⁴⁻¹⁷. This assessment was confirmed using a polarizing filter to highlight the collagen fibers' orientation to reflect the shape of the cement line underling the label with toluidine blue. The RBF and MBF were separately quantified as a percentage of total SbMF surfaces¹⁴⁻¹⁶. The quiescent surface (SbQS) was measured as the percentage of no RBF and no MBF (not stained with toluidine blue), calculated as Ominsky suggested with 100-MBF+RBF+ES/BS¹⁶. The histomorphometry dynamic parameters of bone formation measure the mineralizing surface/BS (SbMS/BS); the mineral apposition rate (SbMAR); the osteoid maturation time (Omt); the mineralization lag time (MLT), and the bone formation rate/BS (SbBFR/BS). Resorption histomorphometry bone parameters (sutural bone osteoclast surface/BS [SbOcs/BS] and sutural bone eroded surface/BS [SbEs/BS]) were quantified as well^{11,13,14}.

These histomorphometric studies were validated with the previously mentioned software comparing control versus PGE₁ groups. The means and standard deviations were calculated in each subgroup. Parameter differences were tested with the Student's t-test and the

Table 1. Radiologic results of palate disjunction and teeth separation

Radiological results	Date	Control group	Sham group	PGE ₁ group	p-values (sham vs. PGE ₁)
X-ray 1 Disjunction (mm)	Basal	0.07 ± 0.006	0.09 ± 0.01	0.08 ± 0.009	0.1
	Day 3	0.09 ± 0.01	0.29 ± 0.05	0.37 ± 0.09	0.05*
	Day 22	0.09 ± 0.01	0.25 ± 0.04	0.24 ± 0.07	0.1
X-ray 2 Teeth separation (mm)	Basal	0	0	0	—
	Day 3	0	4.6 ± 0.56	4.65 ± 0.45	0.1
	Day 22	0	4.33 ± 0.58	5.14 ± 0.34	0.01**

*p<0.05; **p<0.01.

PGE₁: prostaglandin E₁.**Table 2.** Blood and urine bone formation markers on day 22 in rabbits with palate bone disjunction treated with PGE₁

Variables	Control group	Sham group	PGE ₁ group	p-values (sham vs. PGE ₁)
Ca (mg/dL)	11.55 ± 0.72	11.55 ± 0.68	11.85 ± 0.42	0.1
Mg (mg/dL)	2.40 ± 0.27	2.01 ± 0.54	2.20 ± 0.20	0.1
P (mg/dL)	5.17 ± 0.65	5.03 ± 0.43	5.26 ± 0.14	0.1
AkPh (IU/L)	73.35 ± 24.3	67.37 ± 17.0	61.37 ± 11.2	0.1
Bone AkPhO (%)	53.12 ± 10.5	56.50 ± 9.38	50.00 ± 16.2	0.1
Vitamin D ₃ (ng/mL)	38.16 ± 15.7	47.0 ± 20.6	39.0 ± 18.7	0.1
PTHi (pg/mL)	28.1 ± 15.6	30.2 ± 26.0	27.4 ± 10.1	0.1
Calcitonin (pg/mL)	32.3 ± 11.3	40.1 ± 20.5	28.4 ± 15.1	0.1
Calcitriol (pg/mL)	50.5 ± 13.15	48.0 ± 13.8	82.1 ± 13.5	0.002**
Urine bone formation markers on day 22				
Ca (mg/dL)	186 ± 665	178 ± 53.3*	376 ± 102.2	0.001*
P (mg/dL)	28 ± 5.3	29 ± 14.1	42 ± 21.6	0.1
N-telopeptide (nMBCE)	32 ± 4.1	38 ± 16.5	30 ± 2.0	0.1

*p<0.001; **p<0.002.

AkPh: total alkaline phosphatase; AkPhO: alkaline phosphatase thermolabile bone fraction; PGE₁: prostaglandin E₁; PTHi: intact parathyroid hormone.

Fisher test (with a significant value of < 5%). All results were analyzed with the SPSS V-18 software.

Results

Palate bone disjunction and *in vivo* radiological results are shown in **Table 1**. On day 3, we observed a significant difference of palatine suture width ($p < 0.05$) between the sham and PGE₁ groups. On day 22, a significant teeth separation ($p < 0.01$) was detected between these groups.

Blood levels of Ca, Mg, and P were similar between groups. The absence of hypercalcemia in the PGE₁ group was noticeable (**Table 2**). No significant differences were observed in biochemical markers concentration, except for calcitriol in the PGE₁ group, which was significantly higher than the sham group ($p < 0.002$).

Urine Ca levels group were significantly high ($p < 0.001$) in the PGE₁, but the phosphaturia and the cross-linked N-telopeptide of type 1 collagen were similar in all groups. Interestingly, the biochemical marker

of bone resorption (N-telopeptide) was not increased in the PGE₁ group (**Table 2**).

The structural histomorphometry parameters of the sutural bone in the PGE₁ group exhibited anabolic bone formation evidenced by significant increases in the SbAr ($p = 0.014$), SbTh ($p < 0.001$), and SbV ($p = 0.007$) compared with the sham group (**Fig. 2**). In the PGE₁ group, the static bone formation parameters showed a significant decrease in osteoid thickness, sutural bone osteoid surface, and sutural bone osteoid volume/BV (**Table 3**). The number of sutural bone osteoclast surface/BS was similar in all groups.

In the sham group, we observed 19.7% of SbRBF but only 5.9% of SbMBF. On the contrary, in the PGE₁ group, we observed 8.6% of SbRBF and 16.6% of SBMBF (**Table 3**).

Under polarized light, we corroborated the irregular lamellar lines of RBF and the regular lamellar lines of SBMBF (**Fig. 3**). The sutural surface of quiescent bone areas was similar between the sham and PGE₁ groups (**Table 3**).

Dynamic histomorphometry parameters of sutural bone formation showed differences in mineralizing bone markers. When comparing the control group versus the PGE₁ group, we observed a significant shortening of Omt ($p = 0.009$) and mineralizing lag time ($p < 0.001$) (**Table 3**). The SbBFR/BS activity were similar between the sham and PGE₁ groups but higher than the control group. With Masson-Goldner trichrome stain, no significant differences were observed in the static histomorphometry resorption parameters of the sutural bone between groups (**Table 3**).

Discussion

The present study demonstrated that PGE₁ treatment produced a wider palate disjunction with fast anabolic sutural bone formation, significant reduction of the mineralizing time, and slight bone resorption, resulting in histomorphometry increased BV mainly through modeling-formation with no hypercalcemia. Significant increases in bone mineral volume and fracture risk reduction have generated much enthusiasm for using anabolic therapy in osteoporotic patients.

In the present study, the anabolic impact of PGE₁ administration on the palatine-bones was confirmed by biochemical and histomorphometry findings. PGE₁ increased significantly the synthesis of 1,25(OH)₂D₃ ($p < 0.002$) compared to the control and sham groups but did not produce hypercalcemia, even though hypercalciuria ($p < 0.001$) was detected in rabbits that

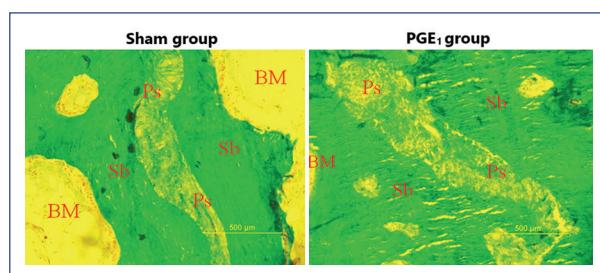


Figure 2. Comparing the sutural bone volume/tissue volume from the sham group versus the prostaglandin E₁ (PGE₁) group. On the sagittal sections with the same magnification for both images, we observed a significant anabolic increase of the sutural bone (right) in the PGE₁ group ($p = 0.007$). Ps: palatine suture; Sb: suture bone; BM: bone marrow. Stained with Goldner's trichrome, 10.0 mm × 12.5 mm objective.

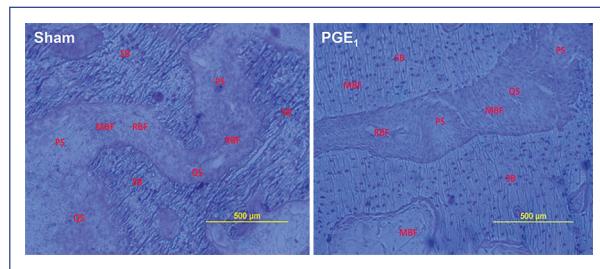


Figure 3. Mineralization front of the sutural bone was identified with a toluidine blue stain. In the sham group (no prostaglandin E₁ [PGE₁]), the scalloped cement lines predominated (remodeling-based bone formation). In contrast, in the PGE₁ group, the smooth cement lines predominated (modeling-based bone formation). MBF: modeling bone formation; PS: palatine suture; QS: quiescent surface; RBF: remodeling bone formation; SB: suture bone. Toluidine blue stain, 10.0 mm × 12.5 mm objective.

received PGE₁ (**Table 2**). We previously observed this phenomenon *in vivo* in rabbits and *in vitro*⁵. The resorption markers (N-telopeptide levels) remained similar due to an apparent low PGE₁ resorption (**Table 2**).

The histomorphometry study on the sutural bones exhibited a significant anabolic growth characterized by a significant increase of the sutural area ($p < 0.01$), sutural thickness ($p < 0.001$), and the SbV ($p = 0.007$) compared with the sham group^{16,18}.

When comparing the histomorphometry parameters between the sham and PGE₁ groups, we found that bone formation was reversed. The RBF in the sham group was significantly higher (SbRBF = 19.7%) when

Table 3. Structural histomorphometry parameters of the sutural bone

Variable	Control group	Sham group	PGE ₁ group	p-values (sham vs. PGE ₁)
SbWi (μm)	125.36 ± 38.79	201.32 ± 96.91	260.08 ± 66.85	0.1
SbAr (mm ²)	0.29 ± 0.12	0.36 ± 0.09	0.53 ± 0.08	0.014*
SbTh (μm)	452.47 ± 86.14	810.03 ± 373.8	1007.8 ± 246.4*	0.001**
SbV/TV (%)	35.62 ± 11.1	42.23 ± 11.25	57.20 ± 8.12	0.007*
Static histomorphometry parameters of sutural bone formation				
SbOTH (μm)	14.05 ± 1.85	13.65 ± 2.02	7.98 ± 2.79	0.004*
SbOS/BS (%)	38.35 ± 10.04	20.12 ± 7.56	8.71 ± 5.10	0.01*
SbOV/BV (%)	0.45 ± 0.27	0.67 ± 0.36	0.26 ± 2.26	0.007*
SbObS/BS (%)	18.62 ± 8.01	19 ± 6.45	15.45 ± 2.60	0.345
SbRBF (%)	13.81 ± 2.85	19.7 ± 6.12	8.64 ± 1.34	0.001**
SbMBF (%)	3.36 ± 0.74	5.98 ± 2.53	16.68 ± 4.42	0.001**
SbQS (%)	81.53 ± 2.96	72.96 ± 7.92	73.84 ± 4.44	0.293
Dynamic histomorphometry parameters of sutural bone formation				
SbMS/BS (%)	67.671 ± 13.56	281.85 ± 56.37	273.07 ± 54.61	0.1
SbMAR (μm/day)	2.85 ± 0.49	4.73 ± 1.13	4.81 ± 0.77	0.983
Omt (days)	5.11 ± 1.35	3.00 ± 0.69	1.72 ± 0.77	0.009*
MLT (days)	23.30 ± 4.03	22.21 ± 3.18	10.38 ± 2.95	0.001**
SbBFR/BS (μm ³ /μm ² /year)	703.94 ± 140.78	4865.99 ± 973.19	4794.15 ± 958.33	0.1
Static histomorphometry parameters of sutural bone resorption				
SbOcS/BS (%)	1.12 ± 0.52	1.34 ± 0.98	0.87 ± 0.38	0.1
SbES/BS (%)	3.63 ± 1.44	4.04 ± 3.15	3.18 ± 1.62	0.1

*p<0.05; **p<0.001.

PGE₁: prostaglandin E₁; SbWi: sutural bone width; SbAr: sutural bone area; SbTh: sutural bone thickness; SbV/TV: sutural bone volume/tissue volume; SbOTH: sutural bone osteoid thickness; SbOS/BS: sutural bone osteoid surface/bone surface; SbOV/BV: sutural bone osteoid volume/bone volume; SbObS/BS: sutural bone osteoblast surface/bone surface; SbRBF: sutural bone remodeling bone formation; SbMBF: sutural bone modeling bone formation; SbQS: sutural bone quiescent surface; SbMS/BS: sutural bone mineralizing surface/bone surface; SbMAR: sutural bone mineral apposition rate; Omt: osteoid maturation time; MLT: mineralization lag time; SbBFR/BS: sutural bone formation activity/bone surface; SbOcS/BS: sutural bone osteoclast surface/bone surface; SbES/BS: sutural bone eroded surface/bone surface.

compared with the MBF of the same group (SbMF = 5.9%). In the PGE₁ group, the histomorphometry bone formation values were contrasting because the MBF was high (SbMF = 16.7%), and apparently, PGE₁ inhibited the activation of bone remodeling (SbRBF = 8.6%) as reflected in decreased sutural bones resorptive parameters (Table 3). Serum biochemical markers were correlated with the histological findings, as the group that received PGE₁ did not exhibit hypercalcemia. No increased resorption marker (N-telopeptides) was found in either the control or PGE₁ groups (Table 2). These findings suggest that PGE₁ differs from other anabolic bone agents and is near to be the “ideal bone anabolic molecule” that could optimize the impact on bone

formation, producing fewer histomorphometry changes in resorption, and not inducing hypercalcemia³.

After Villanueva et al.¹⁴ identified the MF with toluidine blue staining, Miller and Marks¹⁸, using fluorochrome technology, observed that after PGE₁ administration, periosteal bone formation was not preceded by the resorption phase, indicating that bone anabolism is stimulated mainly by bone modeling¹⁹.

At present, the only anabolic bone therapies available are the PTH forms: PTH 1-34, PTH 1-86, PTH 1-36, and abaloparatide^{3,4}. These therapies have proved to be safe and effective. However, they increase bone resorption and induce hypercalcemia. Horwitz et al.²⁰ suggested that PTHr related proteins might increase bone formation in the absence of

hypercalcemia; slight resorption was also observed. Another exciting area in the exploration of combining PTH therapy with antiresorptive agents has produced intriguing results^{21,22}. Some authors used antibodies against inhibitors of bone formation, such as the sclerostin (osteocytes secreted negative regulator of bone formation)¹⁶, odanacatib (cathepsin K inhibitor)²³, and denosumab (a human monoclonal antibody that binds and inhibits RANK)²⁴, and found that these antibodies favored anabolism and did not produce hypercalcemia. Most of these bone inhibitor antibodies, including PGE₁, produced bone anabolism by a mechanism in which the MBF is observed predominately as smooth cement lines. Those changes are significant findings since they may be interpreted as an activation that produces bone anabolism with inadequate bone resorption, usually without hypercalcemia^{14-16,19}.

These findings suggest that PGE₁ treatment could act through an MBF mechanism to achieve anabolism with low resorption and no hypercalcemia in osteopenic diseases. This therapy could be evaluated in clinical research for other diseases, including human maxillary deficiency (a frequent disease in children), osteoporosis in adults, or any osteopenic problem. Our findings may represent new potential information for repairing bone fractures, osteomalacia/rickets, and other osteopenic disorders.

The SbOTH, SbOS/BS, and the SbOS/BV on the PGE1 group were less than the other groups, perhaps due to the acceleration of osteoid mineralization, while the mild increase in the mineral appositional rate is indicative of increased bone production²⁵. Regarding the dynamic bone formation parameters, after 22 days of PGE₁ treatment, we observed a significant shortening of the time of osteoid mineralization and MLT²⁶. These findings suggest that PGE₁ accelerates bone matrix mineralization, decreasing the osteoid sutural BS and increasing bone anabolism.

Understanding the effects of PGE₁ on bone modeling and remodeling might help clarify its effects on bone formation and evaluate bone increase. We observed the same percentage of RBF and MBF in healthy children in iliac crest biopsies of the trabecular bone tissues¹⁵.

The effect of PGE₁ on MBF has not yet been demonstrated in human adults. However, the biomarker's profile and histomorphometry results in children and animal models are encouraging^{14,16,25}. We should further compare the MF identification with staining techniques, like tetracycline chelation.

In conclusion, the exogenous PGE₁ treatment in rabbits exhibited a wider suture palate disjunction, increased calcitriol synthesis, and the sutural palatine bones volume, and shortened MLT. The bone anabolic effect may be exerted mainly through MBF, producing mild resorption without hypercalcemia. These findings could lead to new potential options for osteopenia treatments.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed the regulations of the relevant clinical research ethics committee and those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on patient data publication.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflict of interest.

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