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ORIGINAL PAPER

Homeopathic treatment for bone regeneration: experimental study

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Aim and method: **The objective of this research was to study the effect of homeopathic treatment with *Plumbum metallicum* (*Plumbum met.*) on mandibular bone repair in rats.**
Materials and methods: **We analyzed the mandibles of 60 male rats, approximately 3-month-old, randomly divided into three groups of 20 animals each: control, treated with calcitonin, and treated with a homeopathic medicine. A circumscribed bone defect measuring 4 mm in diameter was made in the mandible and covered with a polytetrafluorethylene (PTFE) barrier. The group treated with calcitonin received 2 IU/kg intramuscularly three times a week; the group treated with *Plumbum met.* 30c received three drops in water every day. The animals were sacrificed after 7, 14, 21 and 28 days. The mandibles were removed and submitted to histologic and histomorphometric analyses.**
Results: **Data were analyzed statistically by two-way ANOVA and by the Tukey test. The interaction effect (ANOVA, F df(6; 48) = 4.64; $p = 0.001 < 0.05$) indicated that the relationship between treatments was not the same at each time of sacrifice. Although statistical analysis of the histomorphometric data showed a similar results for the treated and control groups. But histological analysis showed complete filling of the surgical defect throughout its extent was only for the group treated with *Plumbum met.***
Conclusion: **The study demonstrated that for repair of surgical defects in rat mandibles *Plumbum met.* 30c and control did not differ significantly in histomorphometric terms.**
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Keywords: Calcitonin; bone regeneration; homeopathy; *Plumbum metallicum*

Introduction

The use of homeopathic medicines as inducers of new bone formation is practically absent in scientific publications. Sweeney *et al.*[1] reported the need for alternative methods for the repair of bone defects caused by trauma or resulting from surgical treatment of congenital malformations, neoplasia or infection.

According to Husseman and Wolff[2] in view of the high toxicity of lead, its application is only indicated at homeopathic doses, with its use being recommended in children with deficient bone formation since lead shows marked affinity for calcium. Lead and some other toxic elements are fixed and released by bone in a manner similar to that of calcium.[3] Recent studies on the biological and clinical effects of homeopathic substances have suggested that the information is transmitted to the cells by a potentiated solution.[4]

Fisher *et al.*[5] studied the effect of homeopathically diluted lead on urinary lead excretion in the rat. The homeopathic treatment did not cause a significant change in

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urinary lead excretion compared to distilled water, although there were significant differences between different homeopathic dilutions. The experimental study conducted by Begum *et al.*[4] on rats submitted to lead intoxication demonstrated that homeopathic substances have a significant protective effect on lead-induced subacute hemotoxicity.

Calcitonin is an important regulatory of mineral and skeletal metabolism, interacting with parathyroid hormone in the maintenance of skeletal mass and acting on both bone tissue and calcium homeostasis. Numerous animal studies have demonstrated the action of calcitonin on bone healing.[6–11] In humans, men show approximately twice the high calcitonin levels of women. In contrast, in rats higher levels are found in females.[12]

The possible effect of homeopathic medication on bone repair encouraged the present research to study Plumbum metallicum (*Plumbum met.*) on defects surgically created in rats mandibles.

Materials and methods

Sixty male albino Wistar rats (*Rattus norvegicus*) aged 3 months and weighing 300 g on average were randomly divided into three groups of 20 animals each: control, treated with calcitonin, and treated with *Plumbum met.* 30c. The study was approved by the Local Ethics Committee (07/99-PA/CEP).

After anesthetic, an incision was performed in the skin, followed by muscle dissection and incision of the periosteum. A standard surgical bone defect was created by the same operator in the body of the mandible with the aid of a 4-mm width tungsten carbide bur (Aseptico, Woodinville, WA, USA), activated by a micromotor at 1500 rpm. Physiological saline (0.9%) was used as a cooling agent during the surgical procedure. The bone defect was covered with a polytetrafluorethylene (PTFE) barrier (Brasflon, Tecnoflon, São Paulo, SP, Brazil), positioned both lingually and buccally and exceeding by 3 mm the margins of the defect. The muscles were sutured with absorbable catgut No. 4 thread (Cirumédica, Cotia, SP, Brazil), and the skin sutured with silk No. 4 thread (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil). In the group treated with calcitonin, the animal received injections of 50 IU of synthetic salmon calcitonin (Sandoz AG, Nürnberg, Germany), corresponding to the human therapeutic dose. It was diluted in physiological saline, and 2 IU/kg, was administered intramuscularly on alternate days. In the group treated with homeopathy, the medication was compounding according to the homeopathic pharmacotecnic by Belluz. Lead was triturated in lactose up to 4C by H&N Homeopathic Pharmacy. Of this, 1 drop was added to 99 drops of alcohol 70%(w/w) and the vial succussed 100 times. Each successive potency was prepared with 1 drop of the preceding potency and 99 drops of alcohol 70%(w/w) and succussed 100 times. This was then converted to a liquid potency and further potentisation was carried out by the process of succussion. Each animal received three drops of the medicine per day, added to the water bottle. 7, 14, 21 and 28 days after surgery, five animals from each group were sacrificed. The bone containing the surgical defect was removed *en bloc* and fixed in 10% formalin. The specimens were decalcified in an aqueous 20% formic acid solution (Merck, Darmstadt, Germany), and sectioned in the central region of the bone defect. Samples were embedded in paraffin parallel to the sectioned surface and the blocks were cut into 20 semi-serial sections of 5 μm, which were stained with hematoxylin–eosin and Mallory's trichrome.

Histomorphometric analysis

The histomorphometric analysis was performed by histologists blind to treatment according to a stereologic method based on the technique used by Gomes *et al.*[13] A Carl Zeiss microscope (Zeiss 1.3, Kontron Elektronik, Munich, Germany) equipped with a 40× objective and a color video camera and the KS 400 image analysis system (Kontron Elektronik) were used. The program allowed the elaboration of a square reticule of 105 points. Bone density (Bd) was expressed as percentage and the intersection points between the lines were considered. The reticule images were superimposed on the bone trabeculae (Pi) and the total number of points over the bone defect (P) was counted. The fraction of the area of the bone defect occupied by this structure was calculated by the following formula:

$$\text{Bd} = \frac{\text{number of points over the bone trabeculae (Pi)}}{\text{total number of points over the bone defect } (P)}$$

The reticule was superimposed on the histologic section, representative of the bone defect. The histologic variable considered for quantification (Pi) was bone formation, such as new bone matrix formation. Among the 20 slices obtained from each animal at each observation time, four were randomly selected for histomorphometric analysis.

For statistical analysis, the dependent variable was bone density (Bd). The independent variables were time of sacrifice (7, 14, 12 and 28 days) and treatments (control, calcitonin and *Plumbum met.*).

Results

Microscopic features

7-Day: A significant difference was observed between the group treated with calcitonin and the other two groups. The control and homeopathy showed new bone formation with immature trabeculae surrounded by osteoblasts and smooth connective tissue, whereas the group treated with calcitonin showed a surgical defect area filled with richly cellularized granulation tissue.

14-Day: The control and homeopathy groups, the extremities of the bone defect showed subperiosteal newly formed bone and new bone formation with numerous osteocytes and some osteoclasts. These trabeculae delimited wide marrow spaces filled with connective tissue. In the group treated with homeopathy, the areas of new bone formation showed apposition lines. In the calcitonin group, new bone formation was characterized by immature trabeculae surrounded by osteoblasts.

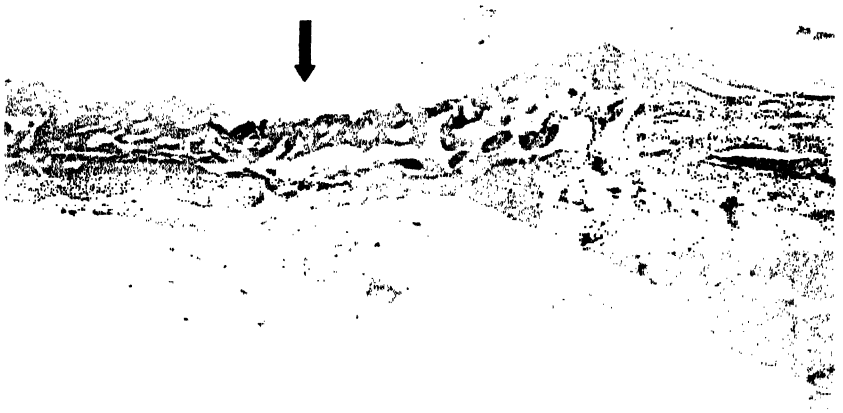

Figure 1 *Plumbum met.* treated group (28-day period): central region of the defect (black arrow) showing the union of the margins by newly formed trabeculae delimiting medullary spaces of variable sizes. (Hematoxylin–eosin staining. Original magnification: 25×).

21-Day: In the control group, the trabeculae were immature, whereas in groups treated with homeopathy and calcitonin greater tissue maturity with inclusion of osteocytes was observed. In the homeopathy group, the trabeculae were intermingled with wide marrow spaces containing either medullary or loose connective tissue. These spaces were smaller in the calcitonin-treated group. Some bone fragments surrounded by osteoclasts and osteoblasts accompanied by the deposition of bone matrix were observed in the group treated with homeopathy.

28-Day: The control group presented mature trabeculae, with osteocytes in a lamellar pattern and osteoblasts and some osteoclasts at the periphery. The entire bone defect was filled with bone tissue intermingled with smooth connective tissue. In the homeopathy group, fusion of the borders of the defect was observed throughout the defect, except for one specimen in which the defect was invaded by muscle tissue. The newly formed bone tissue presented more mature trabeculae than observed in earlier periods, sometimes showing a lamellar arrangement. In the calcitonin group, the new bone formation was more organized, with mature trabeculae presenting reverse lines. In the sections of the innermost portion of the defect however, the presence of granulation tissue was noted in the central part (Figure 1).

Histomorphometric analysis

Descriptive statistics data are presented as means and standard deviations (Table 1). The statistical significance of differences among groups was tested by two-way ANOVA (fixed effect). The interaction effect was observed by plot of means graph (Figure 2). *Post hoc* multiple comparisons were calculated by the Tukey test (Table 2). Statistical significance was defined as $\alpha = 5\%$. The data were analyzed with the Statistix for Windows software (version 8.0, Analytical Software Inc., 2003, FL, USA). Two-way ANOVA applied to Bd data showed that all effects were stati-stically significant. The interaction effect (ANOVA, F df(6; 48) = 4.64; $p = 0.001 < 0.05$) indicated that the relationship between treatments was not the same at each time of sacrifice.

Discussion

Initial filling of the bone defect with blood clots containing bone fragments resulting from the surgical procedure was observed in all groups. At 7 days, discrete bone formation was observed from the periosteum of the region

Table 1 Histomorphometric analysis. Means (±standard deviation) for Bd data (%) obtained for 60 rats by treatment and time of sacrifice

Time of sacrifice (days)	*Treatments*			
	Control	*Calcitonin*	Plumbum met.	*Row (mean ± sd)*
7	85.55 ± 3.93	72.25 ± 6.65	87.00 ± 3.05	81.60 ± 8.18
14	88.25 ± 3.46	76.70 ± 3.56	85.70 ± 6.62	83.55 ± 6.77
21	94.70 ± 1.72	88.65 ± 2.15	93.35 ± 1.80	92.23 ± 3.21
28	93.55 ± 2.38	94.05 ± 1.36	96.65 ± 1.93	94.75 ± 2.28
Column (mean ± sd)	90.51 ± 4.74	82.91 ± 9.73	90.68 ± 5.83	

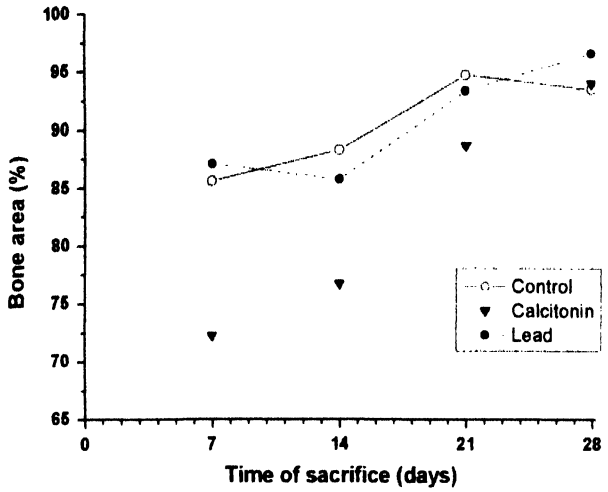

Figure 2 Graphic plot of mean bone area data (%) according to time of sacrifice.

adjacent to the defect. During the other periods, the clot was gradually replaced with granulation tissue, with progressive increase of newly formed bone.

Bone fragments exhibiting osteoclasts were observed at all time points. On some occasions, these fragments were surrounded by rounded cells resembling osteoprogenitor cells. In the group treated with homeopathy, bone matrix deposition around these remnants was noted after 21 days. These bone fragments probably had an osteoinductive action on the undifferentiated mesenchymal cells of the region, which was favored by the microenvironment created by the use of the physical barrier that provides an appropriate concentration of proteins and growth factors which stimulate osteogenesis.[14-19]

The presence of osteoclasts observed in all three groups during the different periods shows that bone remodeling occurs simultaneously to the repair process.[12] This fact became also evident by the presence of reverse basophilic lines during the last observation periods.

Table 2 Tukey test of bone density *vs.* time point ($p < 0.005$)

Experimental condition		*Mean Bd (%)*	*Homogenous grouping**				
Time of sacrifice (days)	*Treatment*						
7	Calcitonin	72.25	A				
7	Control	85.55		B			
7	*Plumbum met.*	87.00		B	C	D	
14	Calcitonin	76.70	A				
14	*Plumbum met.*	85.70		B	C		
14	Control	88.25		B	C	D	
21	Calcitonin	88.65		B	C	D	
21	*Plumbum met.*	93.35		B	C	D	E
21	Control	94.70				D	E
28	Control	93.55			C	D	E
28	Calcitonin	94.05				D	E
28	*Plumbum met.*	96.65					E

* Mean values followed by the same letter are not significantly different.

Complete filling of the defect in the more central region was not observed in any of the specimens from the control or calcitonin-treated group. Although in the group treated with homeopathy, the defect was filled to some extent, reconstitution of the full thickness of bone tissue was not observed. Interposition of muscle tissue in the region of the surgical defect was noted in one animal of the 28-day period, preventing new bone from filling the central area of the defect. These findings confirm those reported by other authors who demonstrated that the interposition of non-osteoprogenic cells in a surgical bone defect hinders the migration of cells with osteoprogenitor potential, thus interfering with the repair process.[14,20-23]

Although statistical analysis of the histomorphometric data showed a similar behavior for the control and treated groups, in histologic analysis differences were observed between the two treated groups. The histologic aspect of the newly formed bone tissue presented more mature trabeculae, sometimes showing a lamellar arrangement. Complete filling of the surgical defect throughout its extent was only observed for the group treated with *Plumbum met.*[20]

Calcitonin has been used for many years as an inhibitor of bone resorption in the treatment of osteoporosis in postmenopausal women.[24] In addition, *in vitro* and *in vivo* studies have shown that this hormone stimulates the growth of bone tissue.[8-11,16,25] Farley *et al.*,[26] studying the *in vitro* effects of calcitonin on cells from neonatal mouse calvaria, observed an increase in the number of osteoblasts that would result in increased bone formation.

Although some experimental studies have shown a favorable effect of calcitonin on surgical defect healing in ovariectomized and non-ovariectomized rats,[8-11,16,25] the same was not observed in this study. In the present investigation, both morphologic and histomorphometric analysis showed lower levels of bone formation in animals treated with calcitonin compared to the other groups.

Aires[12] reported that in humans the level of calcitonin in females is lower than in males, the opposite observed among rats. We cannot state that this is the determinant factor for the difference between the present results and those reported by Arisawa *et al.*,[6] indicating the need for further studies to clarify this point.

Conclusion

The present study demonstrated that for guided bone repair of surgical defects performed in the mandible of male rats the group treated with homeopathic medication *Plumbum met.* 30c and the control group yielded similar results.

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