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Effect of diphosphonate on the prevention of X-radiation-induced inhibition of bone formation in rats

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A histologic and histometric study of the effect of EHDP (ethane-1-hydroxy-1,1-diphosphonate) on the alveolar wound healing of irradiated animals is presented. This drug was selected based on its osteosclerotic effect when applied at low doses. The three left mandibular molars of 80 male Wistar rats were extracted. The rats were grouped into 4 sets of 20 rats each. Group I received no further treatment; Group II received daily intraperitoneal injections of EHDP (7.5 mg/Kg of body weight during 10 days); Group III: 15 Gy of X-radiation were applied to the head; and Group IV were irradiated and injected with EHDP as were animals of Groups III and II. Half of the animals were killed after 14 days and the other half 30 days post-extraction. Sections were made at the level of the mesial socket of the first molar in a bucco-lingual orientation, on which histometric determinations of bone activity were made. The results showed the attenuation of the inhibitory effect of radiation on bone formation when the animals were treated with EHDP.

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It is well known that X-radiation provokes a delay in alveolar wound healing (1–3) due to a diminished bone formation rate (4). Alveolar wound healing is a key issue in those cases in which radiation is necessary for treatment of head and neck cancer. To avoid further complications the removal of those teeth with doubtful prognoses is recommended before irradiation. Previous findings (4) suggest it may prove beneficial to irradiate once the socket has started to fill with bone.

The prevention of a delay of the onset of radiation therapy would be of upmost importance. Thus the aim of this experiment was to find a way to prevent the inhibiton of bone formation caused by X-radiation.

Since there are some data in the literature indicating that ethane-1-hydroxy-1,1-diphosphonate (EHDP) stimulates osteoblastic activity (6, 7) this compound was chosen to speed up bone healing.

Material and methods

Eighty male Wistar rats each weighing 90 g, anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital (0.025 g/Kg of body weight). The 3 left mandibular molars of these animals were extracted with enamel hatches without suture, following the surgical technique of Guglielmotti et al. (5). After surgery neither special diet nor antibiotics were given. The animals were grouped into 4 sets of 20 rats each. Group I received no further treatment and served as a control: Group II received 10 daily IP injections of 7.5 mg/kg of body weight of saline solution of EHDP; Group III received 15 Gy of X-radiation to the head; Group IV were irradiated under the same conditions as Group III and received treatment identical to those of Group II, with the first injection given immediately after the irradiation.

For head irradiation the animals were anesthetized, placed on a polyestyrene plate in supine position and their bodies shielded with a 3 mm lead plate thus exposing the head. Irradiation was given in a single section with a dose of 15 Gy under the following conditions: 220 Kv, 14 mA, rate 330 rads/min, focus-object distance 25 cm.

Half of the animals of each group were killed on Day 14 and the other half 30 days post-extraction always by ether inhalation.

The left mandibles were dissected and radiographed with a conventional dental X-ray apparatus on mammagraphic film. Their vestibular plate was focused (focus-object distance of 25 cm). Thereafter they were fixed in 10% formalin solution, decalcified in ethylene-diaminetetraacetate (pH 7.2) at room temperature for 3 weeks and embedded in paraffin.

Sections were obtained at the level of the first molar mesial socket in a bucco-lingual orientation (8) and stained with hematoxylin-eosin.

Histometric measurements based on standard stereologic methods (9–12) were made on projections of the sections with the aid of an image analyzing system (Kontrol MOP AM 03). Zones

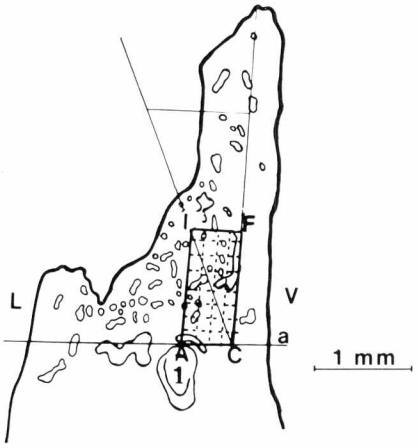

Fig. 1. Drawing corresponds to an alveolus 14 days post-extraction. Bone density was measured in the quadrilateral outlines in the zone which corresponds to the apical third of the socket. It was determined as follows: Line *a*, tangential to the upper cortical border of the mandibular canal (A) and perpendicular to the external surface of the buccal plate; C, the point on line *a* equidistant from A and the external surface of the buccal plate; F, the point one third of the distance from C to the top of the vestibular crest; a line parallel to line *a* from point F on which distance AC is transported determining point I. 1, mandibular canal; V, vestibular plate; L, lingual plate.

of bone formation and bone resorption areas observed microscopically were transferred to the corresponding zones on the tracings. The following histometric determinations were performed following procedures previously reported (5, 8).

Volume density of trabecular bone (VD) considered as the ratio betwen the trabecular area and the bone area, measured in a quadrilateral outlined in the apical third of the alveolus as was reported previously (5, 8) and is explained in the legend to Fig. 1;

Total alveolar volume (TAV) considered as the bone tissue and tis marrow spaces;

Percentage of bone formation area (BF) of the total trabecular bone surface considering bone formation surfaces as those areas covered by an osteoid band with cuboidal osteoblasts present on the bone surfaces of marrow spaces;

Percentage of bone resorption area (BR) of the total trabecular bone surface, considered as the erosive zones present on the bone surface of marrow spaces with or without osteoclasts.

TAV, BF and BR were measured in the area which lies above line *a* which is the tangent to the upper cortical portion of the mandibular canal and is perpendicular to the external surface of the buccal plate (Fig. 2).

The histometric values obtained were analyzed by comparing the data obtained from each group by means of Duncan's orthogonal contrast test and Tukey's w-procedure (13).

Results

The radiographs obtained from animals killed 14 days post-extraction (Fig. 3) showed that in controls, (Group I) the socket was almost completely occupied by radiopaque tissue. Group II afforded similar images. Irradiated animals (Group III) exhibited a radioluscent image of the alveoli. Animals from Group IV showed a delicate radiopaque image in the area corresponding to the apical third of the alveoli.

In those animals killed 30 days post-extraction (Fig. 4) virtually no differences between animals from Groups I, II and IV were observed. Their edentulous areas had been replaced by radiopaque tissue making the socket walls indistinguishable. In irradiated animals (Group III) the edentate ridge afforded a radioluscent image and the only radiopaque image was observed in the apical fundus.

The histologic sections from animals killed 14 days post-extracxtion revealed that in the controls (Group I) the alveolus was almost completely occupied by woven bone. Its trabecular surfaces showed an intense osteoblastic activity while the mucosa completely covered the wound surface.

Animals from Group II afforded a histologic image similar to those of Group I.

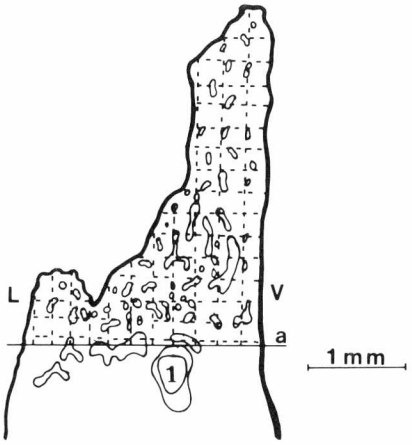

Fig. 2. The drawing corresponds to an alveolus 14 days after the extraction. The dotted zone (above line *a*) represents the area considered for the measurements. 1, mandibular canal; V, vestibular plate; L, lingual plate.

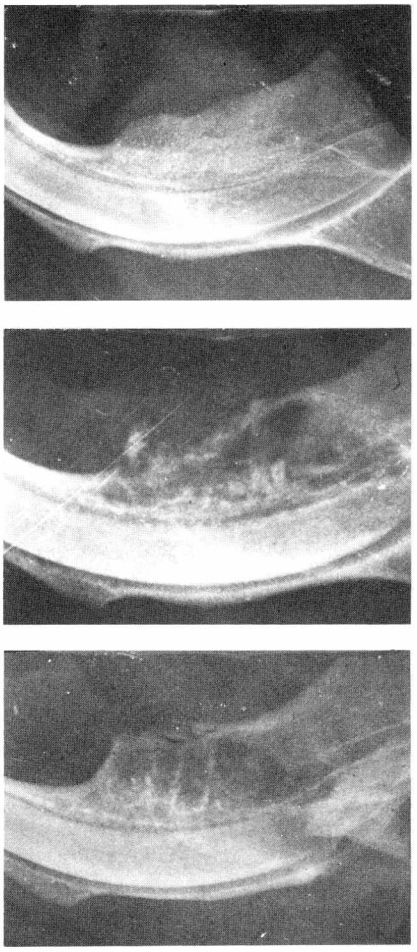

Fig. 3. Radiographic appearance 14 days post-extraction. Upper: control, medium: irradiated; lower: irradiated and injected with EHDP.

The alveolus in irradiated animals (Group III) was occupied by granulation tissue. Few trabeculae with scarce bone forming activity were present in the alveolar fundus. Alveolar plates were very thin and presented many erosive surfaces. No epithelium covered the wound surface (Fig. 5).

Animals from Group IV presented granulation tissue and reticular bone in the fundus of the socket and on the internal surface of the vestibular wall. The wound surface was totally occupied by epithelium (Fig. 6).

The histologic image 30 days post-extraction showed the socket filled by young bone in Group I and Group II animals. Intense osteoblastic activity was present on the bone surface of the alveolar ridge. The sockets of animals from Group III (only irradiated) were full of granulation tissue and only in the

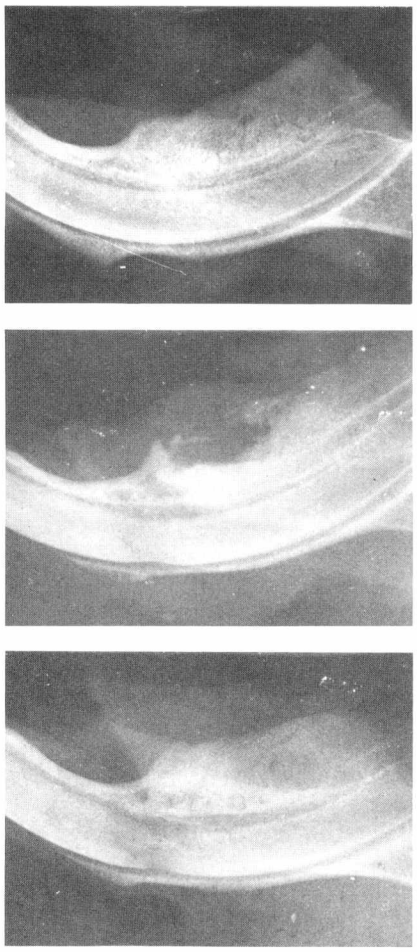

Fig. 4. Radiographic appearance 30 days post-extraction. Upper: control, medium: irradiated; lower: irradiated and injected with EHDP.

lingual sector of the apical fundus newly woven trabeculae with scarce osteoblastic activity were present.

Animals from Group IV exhibited similar histologic characteristics to those of Groups I and II with an alveolus almost totally occupied by young bone and covered by cuboidal osteoblasts. The epithelium completely covered the wound surface in all the groups.

Histometric values obtained from animals killed 14 days after extraction (Table 1) corresponding to TAV, VD and BF areas were lower in Group III than in Groups I and IV. The differences were statistically significant. The values corresponding to TAV, VD and BF are higher in Group IV than in Group III but do not reach the values of Group I. The values of BR were higher in Group III than in Group I and II. These differences were statistically significant, while no significant differences were found between Groups III and IV.

Animals from Group IV killed 30 days post-extraction afforded values similar to those of animals from Group I killed 14 days post-extraction.

As for animals killed 30 days post-extraction (Table 2) BF and TAV values exhibited the greatest differences, with Group IV values much higher than those of Group III (statistically significant).

For both post-extraction conditions Tukey's w-procedure showed statistically significant differences for TAV, VD and BF between Groups I and IV (Tables 1 and 2).

Discussion

The results indicate that EHDP attenuates the inhibitory effect of X-radiation on bone healing when injected at low doses.

The accuracy of the methodology used in this experiment has been repeatedly verified both for the procedure of tooth extraction (5, 8) and for the histometric methods (5, 8–12). In the first case an atraumatic surgical technique was employed (5), which allows tooth extraction without post-operatory complications. The histometric methods are based on standard stereologic procedures and are employed to accurately assess bone activity (9, 10, 12, 14). This methodology was employed in the study of alveolar wound healing after X-radiation affording a numerical characterization of such process (4, 15).

Diphosphonates are drugs used in the treatment of osteoporotic diseases, since they are capable of preventing osteoporosis (6, 17, 18). When given at low doses EHDP increases bone density (7, 16). High doses induce a degree of production of osteoid tissue similar to that found in osteomalacia (16, 19, 20). The new bone formed under EHDP therapy for Paget's disease is of a more normal lamellar structure (19). This effect was attributed to bone resorption inhibition (17, 18, 21). However, some reports indicate an action of EHDP on bone formation (6, 7). This

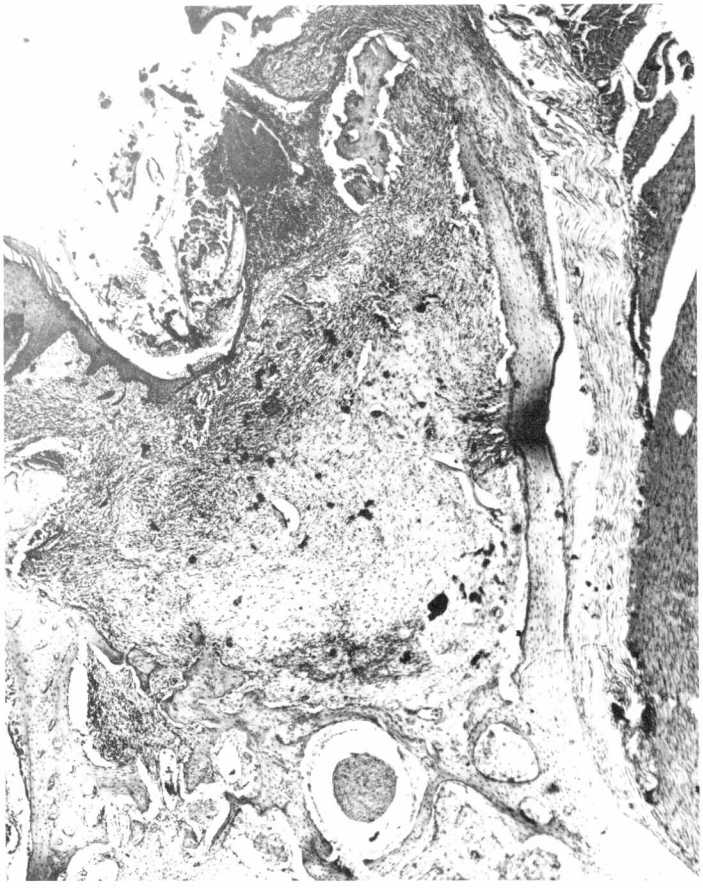

Fig. 5. In irradiated animals the alveolus was almost completely occupied by granulation tissue at 14th day post-extraction (H & E, × 40).

HISTOMETRIC STUDY IN 14 th DAY HEALING SOCKETS

	GROUP I	GROUP II	GROUP III	GROUP IV	COMPARISONS *	HSD **
TOTAL ALVEOLAR VOLUME in μm^2	$15 \times 10^5 \pm 0.6$	$17 \times 10^5 \pm 0.6$	$4 \times 10^5 \pm 0.6$	$8 \times 10^5 \pm 0.7$	I vs III $p < 0.005$ I vs II $p < 0.005$ III vs IV $p < 0.005$	1.00
VOLUME DENSITY Trabecular area/ bone area	0.40 ± 0.03	0.45 ± 0.03	0.11 ± 0.03	0.35 ± 0.04	I vs III $p < 0.005$ I vs II $p < 0.005$ III vs IV $p < 0.005$	0.05
PERCENTAGE OF BONE FORMATION	19 ± 1	19 ± 2	7 ± 1	14 ± 2	I vs III $p < 0.005$ I vs II N.S. III vs IV $p < 0.005$	3.00
PERCENTAGE OF BONE RESORPTION	2 ± 1	1 ± 1	5 ± 2	6 ± 3	I vs III $p < 0.005$ I vs II N.S. III vs IV N.S.	3.00

All values ± S.D.

* Orthogonal contrast

** Tukes's w-procedure (5%)

HISTOMETRIC STUDY IN 30th DAY HEALING SOCKET

	GROUP I	GROUP II	GROUP III	GROUP IV	COMPARISONS *	HSD **
TOTAL ALVEOLAR VOLUME in μm^2	$22 \times 10^5 \pm 2$	$23 \times 10^5 \pm 2$	$6 \times 10^5 \pm 1$	$15 \times 10^5 \pm 1$	I vs III $p < 0.005$ I vs II N.S. III vs IV $p < 0.005$	2.00
VOLUME DENSITY Trabecular area/ bone area	0.60 ± 0.04	0.62 ± 0.04	0.20 ± 0.04	0.41 ± 0.02	I vs III $p < 0.005$ I vs II N.S. III vs IV $p < 0.005$	0.05
PERCENTAGE OF BONE FORMATION	17 ± 1	17 ± 1	5 ± 1	15 ± 2	I vs III $p < 0.005$ I vs II N.S. III vs IV $p < 0.005$	1.50
PERCENTAGE OF BONE RESORPTION	3 ± 1	3 ± 1	6 ± 4	5 ± 1	I vs III $p < 0.005$ I vs II N.S. III vs IV N.S.	2.80

All values ± S.D.

* Orthogonal contrast

** Tukey's w-procedure (5%)

Fig. 6. In irradiated animals and injected with EHDP the alveolus was partially occupied by reticular bone in the fundus of the socket at 14th day post-extraction. (H & E, × 40).

action is not yet clearly understood. In animals with periodontal disease diphosphonate not only resulted in no bone loss but induces an increase in root ankylosis (22). In previous works in which rats treated with EHDP were submitted to orthodontic forces, we obtained preliminary results which suggest that bone resorption and the number of osteoclasts increase (23). These findings indicate that EHDP action is not limited to inhibition of bone resorption.

In the present experiment the action of EHDP on bone formation is well demonstrated. In this system bone resorption does not play an important role and alveolar healing is achieved by bone formed de novo.

It is well known that X-radiation provokes an altered alveolar wound healing (1–4), an event corroborated by histometric methods (4, 15). In this experiment bone formation occurred earlier in irradiated animals which received EHDP than in those animals in which no further treatment was given.

It has been reported that when irradiation is given immediately after tooth extraction the delay in alveolar wound healing is more remarkable than when irradiation is given 7 days after surgery (4, 15). The values obtained here for VD and TAV in animals irradiated soon after tooth extraction and treated with EHDP were similar to those reported for animals irradiated 7 days after extraction (4, 15).

It is important to establish that EHDP was administered during the post-irradiation period. For this reason its action must not be considered to be radioprotective. EHDP prevention of the radioinduced inhibition of the bone formation must be attributed to another mechanism, which will require further analysis.

The data presented here are of therapeutic interest as the administration of EHDP stimulates bone formation thus allowing for irradiation soon after tooth extraction.

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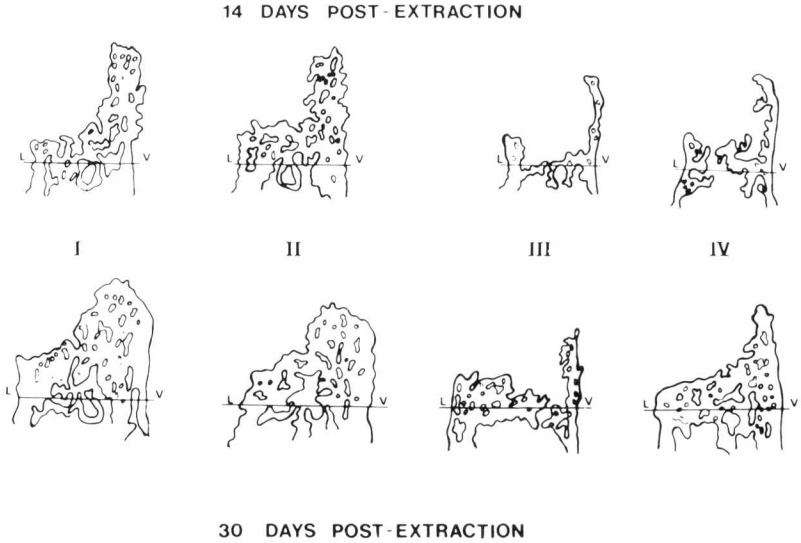

Fig. 7. Tracings obtained by the superposition of the projections of all the sections studied for each time. Note the variation in alveolar volume and ridge modelling between different groups.

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