Histological changes in the bone structure induced at 12 weeks by experimental administration of bisphosphonates

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Abstract: Histological changes in bone structure were induced at 12 weeks by experimental administration of bisphosphonates, which have been associated with osteonecrosis of the jaws. The purpose of this study was to determine the effect of local administration of bisphosphonate on bone formation in rats. Surgically created bone defects were evaluated at 12 weeks by histological examination after bisphosphonate administration. Fifteen Wistar rats that underwent surgery to create a bone defect at the right femur were divided in 3 groups: 2 experimental groups and a control group. The 1st experimental group received bisphosphonate as a single 1-mL dose into the bone defect, the 2nd experimental group received 1 mL of bisphosphonate as a 10-day fractionated dose, and the control group comprised rats who did not receive any bisphosphonate. At 12 weeks after the injection, new bone tissue was collected and a histological examination was performed. At 12 weeks after the bisphosphonate administration, bone repair processes were found in both the experimental and control groups. The bone continuity in the intervention area was ensured by a continuous layer of bone tissue. Compared with the control, the repair processes were significantly more advanced in the experimental groups, the layer of bone tissue covering the bone defect being thicker and more consolidated and the trabeculae from the Haversian canal being thicker and denser. External and internal consolidation structures were present in animals from all of the groups. Bisphosphonate had a positive influence on bone formation. The stimulatory effect of the bisphosphonate increased with the number of administered doses.

Key words: Bisphosphonate, rat, bone remodeling, histology

1. Introduction
There has been an increasing number of reports published on patients with osteonecrosis of the jaws associated with the use of bisphosphonates (1–5).

Although evidence suggests that the duration of and preparation for bisphosphonate treatment are important indicators of risk for jaw osteonecrosis, many guidelines continue to categorize risk according to the route by which the bisphosphonate is administered (6). The mechanism of bisphosphonate action is attributed to its implication of osteoblast activity (7).

The aims of this study were: 1) to describe the histopathologic features at 12 weeks after bisphosphonate administration in a rat model, and 2) to identify the effects of bisphosphonate on bone remodeling.

2. Materials and methods
Following approval from the institutional ethical committee (the procedures and protocol were approved by the institutional review board of the university and by the ethics committee, certificate number 172/2010), we selected 15 Wistar rats, 5 in the control group and 10 in the experimental group. All of the rats underwent surgery to create a bone defect with a 1.5-mm-diameter bone-bur at the right femur transcortical through the Haversian canal. Rats from the experimental group were divided into 2 subgroups: group A, which received zoledronic acid, a commonly used bisphosphonate on the jaw bone, in a 1-mL single dose in the bone marrow during surgery, and group B, which received zoledronic acid in 1-mL divided doses daily at 0.1 mL for 10 days. The control group did not receive any bisphosphonate.

The pieces were fixed in 10% formalin for 7 days and then decalcified in 7% trichloroacetic acid solution, changed several times. They were then inserted into a mixture of 70% alcohol and glycerin for 12 h and then 2 glycerin baths for 24 h each, followed by 2 absolute alcohol baths for 45 min each. Next, they were passed through 2
baths of butyl alcohol for 24 h each and 3 baths of melted paraffin at 56 °C, and finally cast in paraffin blocks. Division of the samples was made with a microtome (Leica Biosystems, Germany) at a thickness of 5 µm; the sections were displayed on a glass slab that was previously coated with a thin layer of Meyer albumin. They were then submitted to 45 °C for 24 h. Probes were colored by Goldner's trichrome method as follows: the slides were decorated in xylene, with 2 changes for 2 min each; placed in absolute alcohol (95% alcohol) with 2 changes for 2 min each; rinsed in distilled water; inserted in Lugol's solution for 5 min; rinsed with distilled water and inserted into a 5% solution of sodium thiosulfate for 5 min; rinsed again with distilled water; placed in Weigert's hematoxylxin for 10 min; washed in running tap water for 10 min; rinsed in distilled water and stained in Ponceau acid fuchsin for 5 min; washed in 1% acetic acid and placed in phosphomolybdic acid–orange G solution until the collagen was decolorized; rinsed in 1% acetic acid for 30 s; stained in light green stock solution for 5 min; and rinsed in 1% acetic acid for 5 min. The slices were dehydrated in 95% alcohol and absolute alcohol with 3 changes each and were then cleared in xylene with 3 changes. A cover glass was mounted with appropriate medium (refractive index: 1.48–1.56). All of the samples were subjected to optic microscopic (Olympus BX41) examination at 12 weeks to evaluate differences in the bone structure.

3. Results
Microscopic examination of serial sections of the intervention performed at 12 weeks after the start of the experiment showed bone repair processes in all of the studied animals. These were still developing, which showed that the intervention area was relatively well consolidated. Pending completion of the repair process, a certain period of time must pass. There were differences between the 2 experimental groups, and between them and the control group, regarding the stages reached by the recovery and consolidation processes of bone repair in the intervention area.

In the control group, the surface area of the defect was occupied by primary bone, which closed the communication with the exterior (Figure 1a), and the Haversian canal was occupied by bone trabeculae in the process of bone restructuring (Figure 2a), the areola between them being occupied by bone marrow. Trabeculae existing in the depth of the bone defect were relatively thick and fully occupied the cavity of the bone defect area (Figure 2b). Areolas from this bone were filled with bone marrow of normal structure. Newly formed bone covered the bone from the surface of the defect area by a thick layer of spongious periosteum bone with tendency of remodeling to compact bone (Figure 2c).

Furthermore, this bone derived from the periosteum extended on the exterior side of the bone in the defect area, covering practically the whole circumference with its thickness decreasing as it moved away from the border of the hole (Figure 2d). In some areas, its thickness was very large, exceeding up to 50% of the wall thickness (Figure 2e) with a spongious appearance on almost 70% of its internal side, showing that it was still in the process of proliferation and remodeling to compact bone.

In experimental group B, the surface area of the bone defect was occupied by the primary bone, which closed the external communication. The Haversian canal was filled with bone trabeculae that were in the process of repair and the areola separated from them contained bone marrow with a typical structure (Figure 3a). The bone trabeculae were relatively thick, in the mid-surface of the defect (Figure 3b) and in the depth of the defect (Figure 3c), indicating that the repair and strengthening of the experimental bone defect were quite advanced, although still not completed. In the animals of this group, the bone wall was doubled by bone periosteum bone from the consolidation process, which disposed its entire circumference, although its thickness was not as great on the whole surface (Figure...
Figure 1. Control group at 12 weeks from the beginning of the experiment (Goldner’s trichrome, 4×): a) 1, the primary proliferated bone at the surface of the experimental bone defect and 2, the bone trabeculae in the Haversian canal; b) 1, the boundary between the existent bone and the newly formed bone at the periphery of the experimental bone defect and 2, the periosteum anchorage bone; c) 1, the periosteum and 2, the connective tissue from the surface area of the experimental bone defect; d) 1, the polymorph bone trabeculae and 2, the areolas partially occupied by bone marrow; e) the periosteum bone.
Figure 2. Experimental group treated with a single dose of bisphosphonate at 12 weeks from the beginning of the experiment (Goldner's trichrome, 2×): a) 1, the defect margins and 2, the bone trabeculae proliferated in the Haversian canal; b) 1, bone trabeculae well anchored in the depth of the experimental bone defect; c) 1, the periosteum bone, primary at the surface and 2, spongious bone in the depth; d) 1, different thicknesses of the periosteum anchorage bone in relation to the distance to the defect; and e) 1, the periosteum anchorage bone occupying more than 50% of the total bone thickness with a, compact bone and b, spongious bone.
In the thickest portion, this bone exceeded 50% of the wall thickness, its structure being compact in the external two-thirds and spongious in the internal third (Figure 3e), suggesting that the processes of bone repair were quite advanced (basically the most advanced from all of the studied animals) but that there was some time needed for completion of bone repair processes.

4. Discussion

In the control group, the bone continuity in the intervention area was assured by a continuous layer of bone tissue, which interrupted the communication with the outside. The spongious bone in the process of proliferation represented the internal consolidation “armor” trying to rebuild bone strength in the intervention area. In the area with the maximum thickness, the periosteum bone collar reached about 30% of the total thickness of the bone wall. Hence, the bone had continuity in the intervention area and some resistance given by the internal and external building structures.

In experimental group A, the defect was covered by a layer of compact bone, which ensured bone continuity in the intervention area. Compared with controls, the repair processes were significantly more advanced, the layer of bone tissue covering the bone defect being thicker and more consolidated and trabeculae from the Haversian canal being thicker and denser. Areolas between the trabeculae were occupied by bone marrow with normal bone structure. The external consolidation components were significantly thicker and exceeded 50% of the total bone wall thickness at its thickest portion. The structure of this bone was spongious (trabeculae) on more than half of the internal side, suggesting that it was still in the process of proliferation and, specifically, remodeling to compact bone.

In group B, the surface area of the bone defect was occupied by a more consistent continuous bone layer with a thickness that was the largest of all of the studied groups. Even if the thickness was not significantly larger compared with the group treated with a dose of bisphosphonate, its structure was found to be in the most advanced remodeling and consolidation stage of all of the studied groups. However, the repair and strengthening processes of the experimental bone defect were not yet finalized.

Following the basics of the bone repair process, we could assess the modality and speed of the repair processes in the animals of all of the studied groups. Although these processes were running somewhat parallel, the restoration of the continuity of the bone wall and the interruption of the communication between the Haversian canal wall and the exterior of the bone represented the highest priority. The layer covering the bone defect was slightly thickened and markedly consolidated in the animals of all of the studied groups, with the differences among groups being largely maintained. The emergence and evolution of the external and internal building structures were slightly different. The external structures enhancing the bone wall occurred relatively early and had the same starting point, the periosteum near the experimental defect.

Based on the bone-forming cells from the inner layer of the periosteum, osteoblasts developed. These were actively involved in bone building that extended in 2 directions: towards the bone defect to form the layer for restoring its continuity and as a thin layer between the periosteum and the compact bone surrounding the defect (like a collar). Proliferation was not identical in the 2 directions; bone that tended to cover the defect proliferated significantly faster than the collar-building bone such that the latter was relatively thin and did not extend the entire circumference of the bone. This periosteum consolidation bone, although it was not very developed yet in any of the studied groups, was different among the groups. The most developed was found to be present in group B, followed by group A and the control group.

The situation was different regarding the strengthening of the internal structures. Animals from the control group showed the development of the internal structure as being in an early stage of consolidation. There were thin strips of bone that penetrated into a certain distance of the internal wall of the experimental bone defect at its periphery (mainly periosteum in origin and partly endosteum). There were also a few contours of short and infrequent trabeculae that did not yet assess stable relationships. This bone represented the early stage of organization of the internal building structures, but did not yet significantly participate in the restoring of the bone wall resistance. The situation was different regarding the experimental groups, which showed polymorphic branched trabeculae (in the process of formation and consolidation), which occupied most of the Haversian canal. There remained only a few bone gaps unoccupied by trabeculae, which were found to be slightly more in group A compared to group B. These bone trabeculae represented the consolidation structure of the defect zone, which was comparable to a scaffold, being organized on the lines of force direction. The existence of internal building structures, which were far more organized in the experimental groups compared with the control group, clearly showed that the bisphosphonate significantly stimulated bone proliferation. In the experimental groups, bone strength was restored to some extent. However, a long time period will be needed until full recovery of the bone resistance is achieved.

External and internal consolidation structures were found in the animals from all of the groups. Thus, external structures were found in the form of a collar bone of uneven thickness, ordered along the entire bone circumference.
Figure 3. Experimental group treated with 10 doses of bisphosphonate at 12 weeks from the beginning of the experiment (Goldner’s trichrome, 10×): a) 1, defect margins, 2, proliferated bone in the experimental defect area, and 3, the periosteum anchorage bone; b) 1, compact bone from the surface of the experimental bone defect, 2, thick bone trabeculae from the surface of the experimental defect, and 3, the polymorph areolas; c) 1, bone trabeculae from the deep area of the experimental defect and 2, areolas with bone marrow; d) 1, the periosteum anchorage bone and 2, the bone wall; e) 1, bone recovery towards compact bone and 2, spongious bone.
Among the groups, there were differences regarding the thickness and degree of bone consolidation. In the control group, the periosteum bone collar was 30% of the bone wall thickness in its thickest area, while it occupied 50% in the experimental groups.

There were no significant differences found regarding the bone thickness between the experimental groups, but structurally, it was a relatively compact bone. In experimental group A, the bone consisted of 30% compact bone and 70% spongious bone, while in experimental group B, the compact bone occupied approximately 70%. These differences showed that the thickness of the periosteum collar will not exceed half of the bone thickness. It will gradually transform into compact bone. The rate of compact bone/spongious bone was in favor of group B. This showed that the stimulatory effect of the bisphosphonate increased with the number of administered doses.

Bone strengthening of the internal structures was present in all of the groups, but there were marked differences in their levels of organization. These aspects suggested that the experimental groups ensured the best mechanical strength. However, the finalization of the postoperative bone healing will take some time.

The bone repair processes occurred normally in all of the studied groups. No pathological processes were reported. This showed that not only was the drilling of the holes done correctly, but other conditions related to surgery were also good (anesthesia, incision, and suture). The natural bone repair process occurred without complications.

Other studies showed that bisphosphonates inhibited bone resorption in vitro (8). The reduction in bone turnover induced by traditional bisphosphonates was explained as being due to the reduction in the extent of osteoblast-covered surfaces (9). Previous evidence has shown that where osteoblasts are present, more bone is made, as indicated by an increase in the wall bone thickness of animals under long-term treatment with bisphosphonates (10–12). Some authors have suggested that zoledronate could be toxic to fibroblasts (13), which we could not verify.

In conclusion, bisphosphonate had a positive influence on bone formation. The stimulatory effect of the bisphosphonate increased with the number of administered doses. The new bone area increased under the influence of bisphosphonate, a single administration shortening the healing bone period compared to multiple dose administration.

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References