Serum changes induced by intramedullar experimental administration of bisphosphonates

H. ALMĂŞAN1, G. BĂCIUŢ1, MIHAELA BĂCIUŢ2, OANA ALMĂŞAN3, S. BRAN2, L. OANA4

1) Cranio-Maxillofacial Surgery
2) Maxillofacial Surgery and Implantology
3) Prosthodontic Dentistry

"Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca
4) Anesthesiology, Propedeutics and Surgical Techniques, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca

Abstract
Bisphosphonates, stable analogues of pyrophosphate, have the ability to bind to bone molecules, possessing anti-resorbtion properties influenced by the radicals linked to the carbon group of their structure. Bisphosphonates link to the hydroxyapatite of the mineral surfaces and are selectively internalized by osteoclasts, whose activity they inhibit, jeopardizing the osteoblastic activity. The purpose of this study is to determine the influence of intramedular administration (at the hip bone) of bisphosphonates on the serum values of alkaline phosphatase, total Ca, proteins and serum osteocalcin in a lot of experience Wistar rats. Fifteen Wistar rats of experience, five in the control group and 10 in the experimental group. All rats underwent surgery to create a bone defect with a 1.5 mm diameter bone-bur at the right femur transcortical through the medullar canal. Rats from experimental group were divided into two groups: group A, who received Zometa 1 mL single dose intramedullar, intraoperative and group B, who received Zometa 1 mL in divided doses daily, 0.1 mL for 10 days. 3 mL of blood from the frontal sinus were collected from each subject at 24 hours, 14 days and 21 days postoperatively. From the blood samples were determined: alkaline phosphatase [U/L], osteocalcin [mmol/dL], Ca2+ [mmol/L, mg/dL], total Ca [mmol/L, mg/dL], osteocalcin [mmol/L]. The data were statistically analyzed using the ANOVA test. We found an increase in alkaline phosphatase [U/L] in all groups studied. In group B there was a significant decrease in total Ca levels [mg/dL] throughout the experiment compared with controls (11.82 → 10.36 → 9.25 mg/dL; 2.95 → 2.59 → 2.31 mmol/L; p=0.001). Ca2+ has changed significantly both in group A (1.18 → 1.25 → 1.25 mmol/L; p=0.01) and group B (1.21 → 1.24 → 1.13 mmol/L; p=0.02). Serum proteins were significantly reduced both in the control group (9.4 → 8.5 → 8.1 g/dL; p=0.03) and the experimental groups (9.3 → 8.5 → 8.3 g/dL; p=0.01) and B (9.9 → 7.6 → 7.3 g/dL; p=0.0008). At each stage of bone development, multiple factors act in a coordinated manner that leads to increased local metabolic processes, acting both on the process of bone resorption and bone repair. Healing processes are initiated within 24 hours in both studied groups and the control group; at 14 and 21 days the bone healing processes are compromised directly proportional to the administration manner and dose of bisphosphonates.

Keywords: bisphosphonates, bone remodeling, bone repair, osteocalcin.

Introduction
Bisphosphonates improve bone strength in animals and estrogenic-deficient subjects by reducing bone turnover and by extending the phase of secondary mineralization of bone remodeling [1–4]. In the literature there are many studies describing the biochemistry, pharmacology, and clinical applications of bisphosphonates medication [5–12]. By 1960, Fleisch H and Neuman WF [13] have studied collagen induced calcification mechanisms and concluded that there are calcification inhibitors in the plasma. It was shown that inorganic pyrophosphate, a natural polyphosphate is present in serum and could prevent calcification by binding to hydroxyapatite crystals [14–15]. Studies on hypo-phosphathes, which is manifested by lack of alkaline phosphatase, show that this is associated with defects of mineralization; manifested by the increase of plasma alkaline phosphatase, this being an enzyme that hydrolyzes pyrophosphate [16]. Bisphosphonates are a class of compounds containing the structure of the P–C–P, which unlike P–O–P structure present in the pyrophosphate, is resistant to chemical and enzymatic hydrolysis [17]. Bisphosphonates are able to bind to bone mineral surface by the chelating ability of calcium ions, and inhibition of bone resorption [5]. By inhibiting protein synthesis, bisphosphonates inhibit the osteoclast activity; which become inactive and undergo the apoptosis process [18, 19]. Bisphosphonates are used to treat and prevent excessive bone resorption mediated by osteoclasts [20–22]. Bisphosphonates are used to treat osteoporosis [23] and bone metastases in prostate and ovarian carcinomas.

In this study, we aimed to determine serum alkaline phosphatase, proteins, total calcium, Ca2+ and osteocalcin in a group of rats Wistar, who underwent a bone defect in the femur, which was inserted with a product based on bisphosphonates (Zometa). We wanted to determine the effect of bisphosphonates on alterations in serum concentrations of compounds, according to the admini-
stration of bisphosphonates or not and depending on
their type of administration.

Materials and Methods

The study group was composed of 15 Wistar rats, male, six weeks old, weighing 100–200 g. The rats were divided into a control group of five rats and an experimental group of 10 rats. The study was conducted over 30 days. Wistar rats were kept for acclimatization in the biobase for a week before the start of the experiment. All rats underwent a right femoral bone defect. After obtaining an aseptic operative field (Figure 1), we performed a line of incision in the thigh (Figure 2); plans were off to reveal skin and muscular and femur (Figure 3). Under continuous cooling, bone notching was performed (Figure 4) using a 1.5 mm diameter bone-bur. An intra-medullar hole at 0.5 cm from the thighbone joint was drilled (Figure 5), in which bisphosphonate was injected to the experimental groups (Zometa) (Figure 6). Group A received a single dose of 1 mL bisphosphonate (Zometa) (Figure 7) intra-medullar, intraoperatively. Group B received 1 mL of bisphosphonate (Zometa) in divided doses, 0.1 mL daily for 10 days. From each subject 3 mL of blood were collected from the frontal sinus, preoperative (T0 time), after 14 days (T1 time) and after 21 days (T2 time) postoperatively. The wound was closed in two planes (muscle and skin) (Figure 8). There have been determinations of blood protein serum [g/dL], alkaline phosphatase [U/L], total Ca [mmol/L], total Ca [mg/dL], Ca²⁺ [mmol/L] and osteocalcin [ng/mL] at time T0 (intraoperative), time T1 (14 days after intervention) and at time T2 (21 days after surgery) (Table 1).
Serum changes induced by intramedullar experimental administration of bisphosphonates

Figure 7 – The bisphosphonate drug used in the study.

Figure 8 – Bone defect closure in two planes.

Table 1 – Serum levels of constants determined during the surgical procedure (T0), after 14 days (T1) and 21 days (T2) in the examined samples

<table>
<thead>
<tr>
<th></th>
<th>T0 (intraoperative)</th>
<th>T1 (after 14 days)</th>
<th>T2 (after 21 days)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Standard Deviation</td>
<td>Mean</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td><strong>Serum proteins [g/dl]</strong> (normal values: 5–8 g/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Sample</td>
<td>9.36</td>
<td>0.610737</td>
<td>8.54</td>
<td>0.581378</td>
</tr>
<tr>
<td>Experimental Sample A</td>
<td>9.28</td>
<td>0.571839</td>
<td>8.52</td>
<td>0.204939</td>
</tr>
<tr>
<td>Experimental Sample B</td>
<td>9.86</td>
<td>0.676757</td>
<td>7.62</td>
<td>0.491935</td>
</tr>
<tr>
<td><strong>Alkaline Phosphatase [U/L]</strong> (normal values: 50–245 U/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Sample</td>
<td>223.8</td>
<td>86.36377</td>
<td>319</td>
<td>120.3266</td>
</tr>
<tr>
<td>Experimental Sample A</td>
<td>273.6</td>
<td>134.2844</td>
<td>308.2</td>
<td>95.2481</td>
</tr>
<tr>
<td>Experimental Sample B</td>
<td>284.6</td>
<td>68.64984</td>
<td>345.8</td>
<td>96.35196</td>
</tr>
<tr>
<td><strong>Total Calcium [mmol/L]</strong> (normal values: 1.75–3.25 mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Sample</td>
<td>11.378</td>
<td>0.626793</td>
<td>11.268</td>
<td>0.686855</td>
</tr>
<tr>
<td>Experimental Sample A</td>
<td>11.134</td>
<td>0.551208</td>
<td>11.152</td>
<td>0.3468</td>
</tr>
<tr>
<td>Experimental Sample B</td>
<td>11.818</td>
<td>0.763492</td>
<td>10.36</td>
<td>0.326726185</td>
</tr>
<tr>
<td><strong>Ca²⁺ [mmol/L]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Sample</td>
<td>1.2</td>
<td>0.04</td>
<td>1.266</td>
<td>0.033615</td>
</tr>
<tr>
<td>Experimental Sample A</td>
<td>1.179</td>
<td>0.04494441</td>
<td>1.254</td>
<td>0.033615473</td>
</tr>
<tr>
<td>Experimental Sample B</td>
<td>1.206</td>
<td>0.035777</td>
<td>1.244</td>
<td>0.02881</td>
</tr>
<tr>
<td><strong>Osteocalcin [ng/mL]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Sample</td>
<td>0.54</td>
<td>0.5029911054</td>
<td>1.08</td>
<td>0.622093241</td>
</tr>
<tr>
<td>Experimental Sample A</td>
<td>2.1</td>
<td>1.228821</td>
<td>2.12</td>
<td>1.878031</td>
</tr>
<tr>
<td>Experimental Sample B</td>
<td>1.16</td>
<td>0.873499</td>
<td>1.38</td>
<td>0.867179</td>
</tr>
</tbody>
</table>

*p<0.05 – statistically significant.

From the blood samples alkaline phosphatase [U/L], serum proteins [g/dL], total Ca [mmol/L, mg/dL], Ca²⁺ [mmol/L] levels were processed. For determination of rat osteocalcin ELISA kit was used (Life Science Inc. USCN., Wuhan, China Lot: 100 610 178, ISO 9001:2008, 13485:2003) (Figure 9).

The data were statistically analyzed using the ANOVA test. The research protocol was approved by the Ethics Committee of the University, according to the principles of the Declaration of Helsinki.

To determine total proteins the biuret reaction principle was used. Cupric ions in alkaline solution react with proteins to form a chelated complex colored in purple. The color intensity of this complex is directly proportional to the concentration of proteins and is measured photometrically at 550 nm with a Hitachi 717 automatic analyzer. Determination of alkaline phosphatase was performed according to the catalisation principle of monophosphate hydrolysis in an alkaline pH. According to the method for determining serum alkaline phosphatase activity recommended by DGKC, p-nitrophenylphosphate substrate is hydrolyzed, releasing p-nitrophenol and inorganic phosphate, magnesium ions act to accelerate the enzyme activity. The increase in absorbance at 405 nm corresponds to the serum alkaline phosphatase activity.

Reagent kit for determining serum alkaline phosphatase level was produced under the DGKC recommendation and consisted of reagent 1 (R1) containing diethanolamine buffer (pH 9.8) and magnesium chloride, and reagent 2 (R2) containing p-nitrophenylphosphate solution, using the Hitachi 717 analyzer.
Evaluation of total calcium (Arsenazo III) was based on the reaction of calcium ions in neutral environment with Arsenazo III, with the formation of a blue complex. The increase of this complex is directly proportional to the concentration of calcium in the sample. Konelab kit containing Arsenazo III (0.2 mmol/L, pH 6.75), Imidazole buffer 100 mmol/L, surfactant, and stabilizers, was used. We used the COBAS MIRA apparatus.

Osteocalcin (bone Gla protein BGP) was determined using an ELISA kit designed to determine the level of osteocalcin (OC) in rats. Plate wells are coated with anti-rat osteocalcin. Standards and samples of serum, plasma or other fluids are added to the wells with polyclonal anti-CB antibody conjugated to biotin. After incubation and washing, we added the avidin conjugated by peroxidase (HRP). Finally, TMB substrate is added to obtain a color reaction proportional to the concentration of OC in the samples. After the suspension of enzyme reaction with S stop solution, optical density is read using an ELISA plate reader at 450 nm. OC concentration in the samples is calculated using the calibration curve with standards established by ELISA reader. This automatically makes up the calibration curve and calculates the samples concentrations based on measured extinction.

**Results**

Following the evolution of alkaline phosphatase in the control group, its growth has occurred between the time T0 and T1 and then decreases between the time T1 and T2, but not below the baseline (Figure 10). Evolution of alkaline phosphatase in the experimental groups A (Figure 11) and B (Figure 12) is different from that of the control group, increasing the time between T0 and T1 values and decreasing the time between T1 and T2, but at the time of T2 values are below the baseline T0. Both proteins decreased in the control group (Figure 13) and the experimental group A (Figure 14) and B (Figure 15) from the time T0 to time T1 and T2, to values below the baseline. The values of total calcium [mmol/L] in experimental groups decreased from time T0 to time T1, and from time T1 to T2 (Figures 16 and 17), final values falling below baseline. Evolution of osteocalcin in the experimental groups A and B show a real difference between groups, between time T0 and T1 were seen in both a decrease and an increase both in group A (Figure 18) and in group B, and T2 values are higher than at the time T0, especially in group B (Figure 19).
Serum changes induced by intramedullar experimental administration of bisphosphonates

Figure 14 – Evolution of serum proteins [g/dL] in the experimental group A, between T0, T1 and T2 (p=0.014).

Figure 15 – Evolution of serum proteins [g/dL] in experimental group B, between T0, T1 and T2 (p=0.0008).

Figure 16 – Evolution of total calcium [mmol/L] in experimental group B, between T0, T1 and T2 (p=0.001).

Figure 17 – Evolution of total calcium [mmol/L] in experimental group A, between T0, T1 and T2 (p=0.001).

Figure 18 – Evolution of osteocalcin [ng/mL] in the experimental group A, between T0, T1 and T2 (p=0.99).

Figure 19 – Evolution of osteocalcin [ng/mL] in experimental group B, between the time T0, T1 and T2 (p=0.03).

Discussion

Normal values of alkaline phosphatase in rats are 50–245 U/L. Alkaline phosphatase of bone origin is the only enzyme secreted by osteoblasts with practical importance for bone pathology and increases in serum when there is a reaction associated with bone formation or repair. For this reason, in humans during the growth period (up to 15–17 years) the activity of serum alkaline phosphatase values is doubled or tripled compared to those seen in adults. Increases of bone alkaline phosphatase occur in rachitic, hyperparathyroidism, Paget’s disease, primary or secondary tumor processes. Decreases in bone alkaline phosphatase serum activity occur in severe vitamin C deficiency, severe hypothyroidism, hypophosphatasia. Generally, isolated increases in serum alkaline phosphatase activity, not accompanied by increases in gamma-GT activity, show increased serum activity of bone isoenzyme. The increase in liver isoenzyme activity gives an indication of the existence of a cholestasis process. In all studied groups of Wistar rats, we found an increase of the amount of alkaline phosphatase in both T1 and T2 time, suggesting the occurrence of bone repair reactions. Although bisphosphonates inhibit the bone resorption activity, in a well-controlled dose that keeps a good balance between bone resorbing and bone repair processes, osteoblasts still appear.

Evolution of alkaline phosphatase in the control group show a bone repair activity during the first 14 days, then there is the tendency towards the baseline regression of the curve. This explains the stabilization of bone repair processes. Administration of bisphosphonates induces slackening of the bone repair processes, bone resorbing being stimulated. This data explains that
administered bisphosphonates induce a decrease in bone remodeling activity, in the present experiment explained by the low values from the original, although the first 14 days there is an initiation of bone repair, and at 21 days, these processes are continuing on a downward slope regression. Comparing group A with group B, we see that when administrating Zometa in the same dose over 10 days, bone repair processes in group B after day 14 have a tendency to plateau, that is a dormancy of the bone processes at a given time. From this, we conclude that administration of bisphosphonates split over a longer period inhibits bone resorption compared with the same dose administered as a single dose.

Normal values of total protein in rats are 5–8 g%. Values lower than 6% of total protein may be found in the nephrotic syndrome, severe hunger, some malabsorption phenomena and cirrhosis. Elevations over 8% were found in chronic inflammation, connective tissue diseases, leukemia and cancer induced hyper-immunoglobulin levels (multiple myeloma, Waldenström’s disease). Changes in plasma protein concentration can occur in case of hyper-hydration or dehydration. In the groups studied at the T0 time, serum protein value was 9.36 g/dL in the control group and 9.28 g/dL in the experimental group A and 9.86 g/dL in the experimental group B. These values were above the average normal value. During the experiment, values fell close to the maximum normal value (8 g/dL) from T0 to T1 and T2, to values below the baseline. Changes in serum proteins are sensitive to T0, T1, and T2, explaining that once bisphosphonate is linked to the hydroxyapatite group in bone, contact is maintained throughout the action bisphosphonate. This link is hard to destabilize, making the action of bisphosphonates a long-term one, and explaining osteonecrosis manifestations at just 3–6 months after cessation of drug administration.

Normal values of total calcium in Wistar rats are 7–13 mg/dL (1.75 to 3.25 mmol/L). Serum calcium level is increased in cases where the calcium is directed in the extracellular compartment from the bone, kidney or intestine: primary hyperparathyroidism and malignancy (multiple myeloma). Low values may be associated with hypoaluminaemia, hypovitaminosis D and hypomagnesemia and hyperparathyroidism or acute pancreatitis. If we question the values of total calcium [mmol/L] in experimental groups, there was a decrease of values from T0 to T1, and from T1 to T2, the final values falling below baseline, indicating that total calcium values were within normality. This decrease in total calcium by the 21st day in two experimental groups shows that bisphosphonates fixes calcium in bones, and in addition, removes it from the other tissue structures.

Osteocalcin (biochemical marker of bone formation process) is the major non-collagen protein of the bone matrix, being synthesized by osteoblasts. After synthesis, it is partly incorporated into the bone matrix, the rest being in the blood stream. In group A maximum values of osteocalcin were recorded in T0 and T1, in contrast to group B, where maximum values were in T2, which explains that a single dose of bisphosphonate administration causes bone repair activity to be maximal in the first 14 days. In group B, recorded peaks are at 21 days, indicating that bone repair occurs only after accumulation of the administered dose in the body. Evolution of osteocalcin in the control group shows a higher increase than the experimental groups (who received doses of bisphosphonates), suggesting the influence of bisphosphonates on bone repair processes, this medication inhibiting bone remodeling if dosage and route of administration are well chosen, to keep a balance between processes of bone resorption and bone formation, permitting a slow bone repair.

Correlating all obtained blood values, we conclude that a single administration of bisphosphonate induces stimulation of bone repair, unlike rats, which received the same dose but divided. The bone repair in rats receiving divided dose is much slower, occurring only after 21 days of use. The period without bone repair exposes subjects to a predisposition to traumatic pathology (fractures). On the other hand, after cumulating doses after 21 days, patients show “bone stiffness”, but susceptibility to pathological bone fractures is much higher due to inhibition of bone resorption and bone formation.

Considering all these aspects, we recommend single dose administration of bisphosphonates for maximum benefits of the pharmaceutical product and minimum adverse effects. This remark is reinforced by the fact that after 14 days of administration, bone modeling and remodeling processes take a near normal course. Bone resorption activity was inhibited after single dose at least 21 days; automatically the resuming of bone forming process activity is close to those previous to administration.

Takahashi K et al. [24], studying the effects of a bisphosphonate in hypercalcemia of Wistar rats, concluded that the effects of taking bisphosphonates are decreasing the level of free calcium in the blood; the maximum effects are visible two days after administration in the blood stream. In the present study, Ca²⁺ [mmol/L] increased in the experimental group A and B between T0–T1 and decreased between T1–T2. Administration of bisphosphonates was intramedular. Total calcium (both in mmol/L and mg/dL) decreased in all study times (T0, T1, T2) in all studied groups. Our results are consistent with those of the above-mentioned study; lasses of bisphosphonates are useful in malignant bone tumors, reducing the risk of bone metastases. Bisphosphonates are useful in preventing metastasis, but irreparably impair long-term bone repair processes.

Giuliani N et al. [25] reported that small doses of bisphosphonates (etidronate and alendronate 10⁻²⁵ to 10⁻²² mol/L) to cultures of murine bone marrow cells from rats increase the osteoblastic potential of bone marrow with positive effects on precursor bone forming cells line. The results of such research, although conducted on cell cultures, are relatively similar to those of the present study, where small doses of bisphosphonates in the first phase is stimulating osteocalcin (secreted by osteoblasts) in the experimental groups. Osteocalcin values in all groups studied are increasing, reflecting an increased osteoblastic activity,
but correlated with inhibition of osteoclastic activity; the “boomerang effect” of activation of osteoblasts after bone resorbing processes is virtually ineffective. Increased osteoblast activity in the body is futile if they are never activated.

In a study on Wistar rats which received bisphosphonate by blood administration and underwent a femoral cavity, Nagashima M et al. [26] reported a delay in the repair of cortical bone, which is apparently related to local reduction of local bone regeneration turnover, suggesting that bone formation following administration of bisphosphonates is bimodal, stimulating the development of osteogenic cells from bone marrow cells in the period immediately following the occurrence of the lesion and inhibiting the differentiation of osteoblasts at a later period in time. In this study, we demonstrated that, despite intense bone forming activity, osteoblasts fail to differentiate and especially do not get to act because osteoclastic processes are inhibited. If there is no bone destruction, there is no bone repair.

Conclusions

At each stage of bone development, multiple factors act in a coordinated manner leading to increased local metabolic processes, influencing both bone resorption and bone formation processes, allowing bone repair if the dose and route of administration are well chosen.

Single administration of a bisphosphonate has shown increased bone repair compared with fractional administration.

Fractioned administration of bisphosphonates over a certain period significantly inhibits bone resorption compared with the same dose administered as a single dose.

Bone repair processes are compromised directly proportional to bisphosphonate pattern and administration dose.

Based on the results of this study, we recommend administration of a single dose of bisphosphonates for maximum advantages of administering the pharmaceutical product and minimum adverse effects.

References


**Corresponding author**

Horea Artimoniu Almășan, PhD student, Department of Cranio-Maxillofacial Surgery, Faculty of Dentistry, „Iuliu Hațieganu” University of Medicine and Pharmacy, 37 Cardinal Iuliu Hossu Street, 400029 Cluj-Napoca, Romania; Phone +40745–287 887, e-mail: almasanhorea@yahoo.com

Received: November 4th, 2010

Accepted: December 10th, 2010