Alendronate enhances antibiotic-impregnated bone grafts in the treatment of osteomyelitis

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Abstract Bisphosphonates are systemic drugs. There is limited knowledge about their effects when applied locally and in osteomyelitis treatment. A prospective longitudinal randomised controlled study was designed in rat tibia to test the efficacy of local or systemically administered bisphosphonates for controlling the osteolytic reactions and possible effects on local infection control. Tibial osteomyelitis was induced in 72 Wistar albino rats with *Staphylococcus aureus* ATCC 25923 strain. Débridement was performed on all rats in all groups. No other treatment was given to the control group. Treatment groups received “plain bone grafts”, “vancomycin-loaded bone grafts”, “vancomycin-loaded bone grafts+systemic alendronate”, “alendronate-impregnated bone grafts” and “vancomycin+ alendronate-impregnated grafts”. Study results were evaluated by swab cultures, radiology, quantitative computed tomography, dual-energy X-ray absorptiometry (DEXA) and histopathology. *S. aureus* was eradicated in groups II and IV by the sixth week. Diaphyseal widening, bone deformation, diaphyseal widening and osteolysis scores were significantly lower ($p<0.05$),

and bone mineral content, density measurements and DEXA scores were significantly higher ($p=0.001$) with alendronate administration. Histology revealed marked osteoblastic activity. Local alendronate interfered with local infection control. Even though local alendronate at the given dose has stronger effects, the possible effects on the local inflammatory process needs to be clarified.

Résumé Les Bisphosphonates sont des médicaments utilisés par voie systémique avec un effet systémique. Nous n’avons aucune connaissance sur leurs effets lorsqu’ils sont utilisés localement, notamment dans le traitement de l’ostéomyélite. Une étude prospective longitudinale randomisée a été réalisée sur le tibia de rat afin de tester l’efficacité de l’administration systémique ou locale des bisphosphonates sur les réactions ostéolytiques et sur de possibles effets sur une infection locale. L’ostéomyélite tibiale a été induite chez 72 rats albinos Wistar avec une inoculation d’un staphylocoque Aureus ATCC 25923. Une mise à plat a été réalisée chez tous les rats, dans tous les groupes. Il n’y a pas eu d’autres traitements dans le groupe contrôle. Les autres groupes ont reçu une greffe osseuse, soit une greffe osseuse imprégnée de vancomycine soit une greffe osseuse imprégnée de vancomycine et avec injection systémique d’alendronate, soit une greffe osseuse imprégnée d’alendronate et enfin une greffe osseuse imprégnée d’alendronate et de vancomycine. Les résultats ont été évalués par cultures bactériologiques, radio, QCT, DEXA et histopathologie. Le staphylocoque doré a été éradiqué dans les groupes II et IV après six semaines. Le gonflement diaphysaire, la déformation osseuse et l’élargissement diaphysaire avec lésions ostéolytiques ont été de façon significative diminués ($p<0.05$), de même que la densité minérale osseuse et le score DEXA ont été significativement plus élevés ($p=0.001$) ceci grâce à l’administration de l’alendronate.

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L'examen histologique a mis en évidence une activité ostéoblastique et l'on peut affirmer que l'administration locale d'alendronate a une influence sur une infection locale. Cependant, l'alendronate administré localement peut avoir des effets importants au niveau du processus inflammatoire qu'il faudra étudier secondairement.

Introduction

Bisphosphonates are a unique class of drugs that inhibit bone resorption. They have an affinity for bone and are incorporated into the crystal structure of hydroxyapatite. They have been clinically used to treat osteoporosis and Paget's disease [13, 18, 21]. During bone resorption, once osteoclasts dissolve these crystals, bisphosphonates are also released, which then inhibit the further activity of osteoclasts [18, 21].

They were recently proposed for use in the prevention of aseptic loosening and osteolysis after joint replacement surgery [8, 20]. Recent studies also have claimed anabolic effects of bisphosphonates on osteoblasts by means of increasing cell proliferation and maturation [8, 9, 21]. Aspenberg et al. [2] suggested alendronate impregnation into bone grafts, and according to their study results, this improved graft resistance to resorption.

On the other hand, a previous publication has shown that alendronate inhibits immune functions of human peripheral blood mononuclear cells (PBMCs) in a dose-dependent manner by selectively inhibiting antigen-presenting cell functions through a mechanism that can be overcome by exogenous interleukin (IL)-1 [19].

Bisphosphonates are systemic drugs. There is a need to test the efficacy of local or systemically administered bisphosphonates for controlling the localised osteolytic reactions.

In order to test the efficacy of local or systemically administered bisphosphonates to control osteolysis in osteomyelitis, and assess possible effects on local control of infection, a prospective longitudinal randomised controlled study was designed in rat tibia. Bone grafts impregnated with (a) vancomycin, (b) vancomycin+alendronate and (c) vancomycin-impregnated bone graft+systemic alendronate application were used as treatment options. Three other groups were designed for control purposes.

Classic treatment of osteomyelitis involves long-term systemic antibiotic administration. To test the effects of alendronate on immune response in a clinically demanding situation, we did not combine a systemic antibiotic in the study protocol. Evaluations were carried out at weeks 3 and 6 in all groups by radiology, dual-energy X-ray absorptiometry, quantitative computed tomography (CT), microbiological swab test and histology.

Materials and methods

Design of study

Seventy-two adult (6 months of age, approximately 250 g) Wistar albino rats were used. Rats were hosted at the Middle East Technical University's Animal Research Facilities, Ankara, Turkey. They were given free access to food and water. All surgical procedures were performed after administration of 60 mg/kg ketamine/10 mg/kg xylazine. Animals were sacrificed by a high dose of thiopental [25]. All procedures were approved by the Ankara Numune Research and Education Hospital Ethics Committee and were in full compliance with Turkish Law 6343/2, Veterinary Medicine Deontology Regulation 6.7.26, and with the Helsinki Declaration of Animal Rights.

In the first stage of the study, osteomyelitis was induced with an inoculation of *Staphylococcus aureus* ATCC 25923 strain into the left tibias of rats. This organism was sensitive to vancomycin and resistant to methicillin. Three weeks after inoculation, development of osteomyelitis was confirmed radiologically. All bones exhibited radiological signs of osteomyelitis. In the second stage of the experiment, treatment of osteomyelitis was carried out according to the pertinent group protocol. Twelve rats were allotted to each group. Six rats from each group were randomly selected and sacrificed for evaluation 3 weeks after surgery. The remaining rats were sacrificed by the end of the sixth week after treatment.

Treatment groups

All rats in all treatment groups underwent curettage and débridement surgery. Rats in group I were left without any bone grafting. In group II, dead space was grafted with plain bone graft; in group III rats were treated with vancomycin-loaded bone grafts. In group IV, vancomycin-loaded bone grafts were used in combination with weekly subcutaneous alendronate at a dose of 240 µg/kg per week. Group V defects were grafted with alendronate-impregnated bone graft. And finally rats in group VI received vancomycin+alendronate-impregnated grafts. Rats did not receive any form of treatment other than that specified in their group protocol.

Preparation of induction material

S. aureus ATCC 25923 was obtained from the Refik Saydam National Hygiene Center, National Type Culture Collection, Ankara, Turkey. Bacterial stock was diluted in 0.95 ml distilled water. The solution was then transferred to blood and eosin-methylene blue (EMB) agar plates and incubated overnight. *S. aureus* colonies were identified [10].

Induction of osteomyelitis

A 1-cm longitudinal anteromedial incision was used to approach the tibia. An intramedullary aperture was created with a 19G hypodermal needle; 0.05 ml of 0.5% (wt/vol) aethoxysclerol was injected through a 0.3×8 mm micro-syringe, followed by 0.05 ml *S. aureus* ATCC 25923 solution inoculation [14, 17].

Preparation of bone grafts

A total of 100 mg alendronate sodium was dissolved in 5 ml sterile water. For alendronate impregnation, 10 g of xenogenic graft was thawed in 10 ml solution containing 1 mg/ml alendronate for 10 min; afterwards grafts were rinsed 3 times for 3 min in saline [2]. Vancomycin impregnation was accomplished by thawing 10 g graft in 10 ml solution containing 100 mg/ml vancomycin for 10 min [23, 24]. Grafts were first immersed in alendronate and then in vancomycin solutions for group VI.

Evaluation of study results

Cultures

Swabs cultures were obtained at week 3 after inoculation and upon completion of the study. They were inoculated on blood and EMB plates. Identification was accomplished with the Mini Api Machine.

Radiological assessment

Anteroposterior views of left tibias were taken at the third week after inoculation and upon completion of the study. Radiographs were graded according to (1) periosteal reaction, (2) diaphyseal widening, (3) osteolysis, (4) bone deformation, (5) sequestrum formation, (6) effusion and (7) soft tissue swelling [1, 16]. Parameters 1–4 were graded from 0–3 (0 absent, 1 mild, 2 moderate, 3 severe) [16]. Parameters 5–7 were judged as 0 or 1. Grading of each criterion was analysed separately for statistical evaluation [1].

Quantitative CT

Density (D) and atomic content (Z) were measured upon completion of the study with a third-generation scanner [11]. Absorption coefficient distribution was obtained for each pixel in a matrix of 320×320 or 512×512.

Dual-energy X-ray absorptiometry

Bone mineral density (BMD) and bone mineral content (BMC) were measured with the small animal software. The instrument was set to 76.0 kV and 150 μ A, collimation was fine, with standard region of interest mode of 0.3×0.3 cm area, and the sample interval was 1/64.

Histopathology

Samples were fixed in 10% buffered neutral formalin and decalcified using 8% formic and 8% hydrochloric acids. Specimens were then dehydrated in increasing degrees of ethanol. Haematoxylin and eosin- and Mallory's trichrome-stained specimens were investigated by light microscope. The region of interest was photographed using a digital camera. Images were analysed to count polymorphonuclear cells, lymphocytes, foam cells, fibroblasts and osteoblasts in the 0.1 mm² area.

Statistical evaluation

Statistical comparisons among groups were done with Kruskal-Wallis and Mann-Whitney tests. Microbiological culture results were assessed with Fisher's exact test. Comparative analysis for histological evaluation was done with Pearson's correlation.

Results

Evaluations were carried out at the end of the third and sixth weeks of treatment in all groups. Evaluation results are summarised in Table 1.

Table 1 Summaries of *p* values at the end of the third and sixth weeks

	Periosteal elevation	Bone deformation	Diaphyseal widening	Osteolysis	Sequestrum	Effusion	Soft tissue swelling	BMC	BMD	D min.	D max.
Third week	0.029	0.296	0.037	0.241	0.479	1.000	0.424	0.014	0.214	0.050	0.130
Sixth week	0.144	0.020	0.004	0.002	0.562	1.000	0.030	0.001	0.001	0.295	0.276

Radiological criteria with *p*<0.05 in the Kruskal-Wallis test were considered significant, and further statistical analysis was performed to determine which treatment group was the source of this difference

Table 2 Significant differences among treatment groups and *p* values with mean ranks according to the Mann-Whitney test are summarised at the third week of treatment

	Periosteal elevation	Diaphyseal widening
Group 1–2	0.016 (7.7–3.30)	0.548 (6.20–4.8)
Group 1–3	0.690 (5.10–5.9)	0.690 (5.90–5.10)
Group 1–4	0.662 (5.5–6.42)	0.686 (5.50–5.10)
Group 1–5	0.393 (3.80–5.67)	0.870 (4.40–4.67)
Group 1–6	0.662 (6.5–5.58)	0.012 (8.6–3.83)
Group 2–3	0.016 (3.30–7.70)	0.690 (5.10–5.90)
Group 2–4	0.009 (3.30–8.25)	0.429 (5.0–6.83)
Group 2–5	0.02 (3.0–7.0)	0.393 (3.9–5.5)
Group 2–6	0.052 (3.0–7.0)	0.052 (8.20–4.17)
Group 3–4	1 (6.0–6.0)	0.537 (5.20–6.67)
Group 3–5	0.571 (4.10–5.17)	0.571 (4.10–5.17)
Group 3–6	0.537 (6.80–5.33)	0.017 (8.6–3.83)
Group 4–5	0.440 (4.58–5.83)	0.905 (5.17–4.67)
Group 4–6	0.485 (7.33–5.67)	0.026 (8.83–4.17)
Group 5–6	0.262 (6.5–4.25)	0.024 (8–3.5)

At week 3, radiological differences were significant with respect to periosteal reaction ($p=0.029$) and diaphyseal widening ($p=0.37$). Periosteal reaction scores for group II (grafted with plain bone graft) were smaller than for the other groups, and diaphyseal widening scores were less severe in group VI (grafted with vancomycin+alendronate-loaded bone graft) than in the other groups (Table 2).

At week 6, radiological findings of bone deformation ($p=0.020$), diaphyseal widening ($p=0.004$) and osteolysis ($p=0.002$) were significantly outstanding among evaluation criteria. Bone deformation, diaphyseal widening and osteolysis were less severe in group VI (Table 3).

Only bone mineral content (BMC) values were significantly different at the third week ($p=0.014$). By the sixth week both BMC and bone mineral density (BMD) values become significant for groups IV (vancomycin-impregnated

bone graft+systemic alendronate) and VI ($p=0.001$). There was no difference in magnitude of this effect in terms of alendronate application route, $p=1$ (systemic versus local alendronate, with combination vancomycin). Control of infection with a local antibiotic was necessary to observe this effect, $p<0.05$ (Table 3, Fig. 1).

Microbiological swab cultures were not significantly different at week 3 (positive culture rates were 40, 100, 20, 20, 33 and 80%, respectively). By week 6, infection was eradicated in groups II (grafted with plain bone graft) and IV (vancomycin-loaded bone grafts+systemic alendronate application). This was statistically significant ($p<0.05$). Positive culture rates were 50, 0.0, 80, 0.0, 60 and 83% respectively (Fig. 2).

According to histopathological evaluation at week 3, the healing process was poor in all groups. Active infection was

Table 3 Significant differences among treatment groups and *p* values with mean rank according to the Mann-Whitney test are summarised at the sixth week of treatment

	Bone deformation	Diaphyseal widening	Osteolysis	BMD	BMC
Group 1–2	0.095 (3.90–7.10)	0.513 (6.0–5.0)	0.690 (6.0–5.0)	0.144 (4.17–7.50)	0.476 (4.83–6.50)
Group 1–3	0.310 (4.5–6.5)	0.151 (6.90–4.10)	0.056 (7.40–3.60)	0.537 (5.33–6.80)	0.662 (5.50–6.60)
Group 1–4	0.111 (3.60–6.75)	0.190 (6.10–3.93)	0.730 (5.30–4.63)	0.01 (3.50–8.50)	0.010 (3.5–8.5)
Group 1–5	0.222 (4.30–6.70)	1 (5.50–5.50)	0.310 (6.40–4.40)	0.177 (4.67–7.60)	0.052 (4.17–8.20)
Group 1–6	0.792 (6.40–5.67)	0.004 (9.0–3.50)	0.004 (9.0–3.50)	0.002 (3.50–9.50)	0.002 (3.58–9.42)
Group 2–3	0.548 (6.20–4.80)	0.222 (6.70–4.30)	0.095 (7.20–3.80)	0.111 (6.75–3.60)	0.052 (5.50–4.60)
Group 2–4	0.730 (4.70–5.38)	0.413 (5.80–4.0)	0.905 (4.90–5.13)	0.029 (2.50–6.50)	0.029 (2.50–6.50)
Group 2–5	0.310 (6.50–4.50)	0.690 (5.0–6.0)	0.421 (6.30–4.70)	0.730 (5.38–4.70)	0.111 (3.25–6.40)
Group 2–6	0.017 (8.60–3.83)	0.004 (9.0–3.50)	0.004 (9.0–3.50)	0.019 (2.75–7.33)	0.019 (2.75–7.33)
Group 3–4	0.413 (4.20–6.0)	0.730 (4.60–5.50)	0.111 (3.60–6.75)	0.016 (3.0–7.50)	0.016 (3–8.33)
Group 3–5	1 (5.60–5.40)	0.121 (4.10–6.90)	0.548 (4.80–6.20)	0.310 (4.40–6.60)	0.151 (4.0–7.0)
Group 3–6	0.126 (7.8–4.5)	0.126 (7.80–4.50)	0.030 (8.40–4.00)	0.009 (3.20–8.33)	0.009 (3.1–8.33)
Group 4–5	0.190 (6.38–3.90)	0.190 (3.63–6.10)	0.413 (5.88–4.30)	0.016 (7.50–3.00)	0.016 (7.80–3.00)
Group 4–6	0.019 (8.25–3.67)	0.038 (8.0–3.83)	0.010 (8.50–3.50)	1 (5.50–5.50)	0.762 (6–5.17)
Group 5–6	0.052 (8.20–4.17)	0.004 (9.0–3.50)	0.017 (8.60–3.83)	0.009 (3.30–8.25)	0.082 (4–7.67)

Fig. 1 Bone mineral content (BMC) and bone mineral density (BMD) measurements at the sixth week from study groups at the 95% confidence interval for the mean. Group I (without any bone graft), group II (plain bone graft), group III (vancomycin-loaded bone graft),

group IV (vancomycin-loaded bone graft combined with systemic alendronate), group V (alendronate-loaded bone graft), group VI (vancomycin and alendronate-loaded bone graft)

observed in group II. Despite the presence of infection, the numbers of foam cells and fibroblasts increased at this time point. All other groups presented average healing. At week 6, bone healing improved when compared to that of week 3. In group I, healing was the poorest; overall histopathology and cell counts revealed that healing with fibrosis was the most dominant feature in the sixth-week group.

Administration of alendronate was found to be effective in prevention of graft resorption. There was a marked increase in osteoblastic activity in the alendronate- and antibiotic-administered groups. Osteoblast numbers were significantly higher in groups IV and VI than in the group grafted with plain bone graft (group II), $p=0.02$ and $p=$

0.01, respectively. These two groups were significantly different from the other treatment groups, $p<0.05$. Local administration of alendronate was more likely to be associated with osteoblastic activity, $p<0.05$. The osteoblast numbers were in correlation with both BMD and BMC values at the sixth week of treatment, $p=0.001$.

Discussion

The purpose of this study was to (a) test the efficacy of local or systemically administered alendronate on control of local osteolysis and (b) observe possible local effects of alendronate

Fig. 2 Percentage of infection at weeks 3 and 6 for the study groups. Group I (without any bone graft), group II (plain bone graft), group III (vancomycin-loaded bone graft), group IV (vancomycin-loaded bone

graft combined with systemic alendronate), group V (alendronate-loaded bone graft), group VI (vancomycin and alendronate-loaded bone graft)

on infection control. The study model produces a progressive osteomyelitis, which is histopathologically similar to chronic human osteomyelitis [17, 25].

We only applied local antibiotic treatment. Classically this is not a standard treatment regimen, and 6 weeks is not a sufficient duration of time for observing long-term progression of osteomyelitis. In addition to débridement surgery, systemic antibiotic administration is required. The lack of systemic antibiotic administration is a limitation of the current study, and it is the most likely explanation for our inability to eradicate resistant bacteriological agents. However, this fact helped us to test the effects of alendronate on immune response in a clinically demanding situation.

Radiologically, alendronate application was associated with lesser degrees of diaphyseal widening at an earlier stage of treatment. By the end of the sixth week, the effect of osteoclastic activity inhibition become more prominent for group VI (grafted with vancomycin+alendronate-loaded bone graft). This was determined by the degree of bone deformation, diaphyseal widening and osteolysis. We think that periosteal elevation, diaphyseal widening and bone deformation are the most important signs of bone infection.

Both BMC and BMD values became significantly higher in the alendronate treatment groups. Contrary to radiological signs, bone density values were similar for both local versus systemic alendronate application (groups IV and VI). However, an increase in bone densities could only be achieved in the presence of antibiotic application (group V versus groups IV and VI). Lagging of radiological signs during the natural course of osteomyelitis may explain why densitometrical studies provided better data than radiology for comparison of local versus systemic alendronate treatment.

Recent studies have claimed anabolic effects of bisphosphonates on osteoblasts by stimulation of proliferation and maturation [3, 8, 21]. We have also observed a marked increase in osteoblastic activity in the alendronate- and antibiotic-administered groups. Local alendronate was more potent than systemic administration. This property of alendronate may be helpful during graft integration and prevention of graft resorption.

Even though we did not note any adverse effects of alendronate on inflammatory response in histological sections, infection rates for group VI were highest. This might be due to an interference with vancomycin elution or the host defense mechanisms. A previous publication has shown that alendronate selectively inhibits monocyte antigen-presenting cell functions through a mechanism that can be overcome by exogenous IL-1 [19].

Osteonecrosis and decreased vascularity prevent delivery of systemically administered antibiotics to desired tissues at required concentrations. Complications and cost are among other drawbacks of systemic antibiotic usage. Various biomaterials have been tried for local delivery of adequate

antibiotic dose to the infection site [4, 7, 15]. Bone grafts have also been used as antibiotic carriers with favourable outcomes [5–7, 22].

The pattern of antibiotic release from cancellous bone resembles the curve of a negative exponential function [23]. Beta-lactams have been shown to lose their antibacterial effect after a few days [22]. The pH of the impregnation fluid affects the amount of antibiotics released. As a polar molecule, vancomycin can form hydrogen bonds. Vancomycin release was found to be influenced by the time used for impregnation and the pH of the medium. It was not affected by increasing the surface area of the graft [5, 24].

Bisphosphonates are proposed for use in the prevention of loosening and osteolysis after joint replacement surgery [8, 20]. Aspenberg et al. [2] suggested alendronate impregnation into bone grafts. Buxton et al. [4] have used a fluoroquinolone-bisphosphonate conjugate to improve topical antibiotic delivery to bone. Oral alendronate itself has been found to have no significant effect on the biomechanical and compositional properties of bone [3, 21].

The above-mentioned studies on drug impregnation into bone grafts were done with commercially available human dry cancellous bone chips [22–24]. In order for us to comply with these studies we preferred to use commercially available bone grafts during our study. These commercial preparations exhibit greatly reduced antigenicity and evoke minimal immune response. The denaturing process also eliminates osteoinductive properties of the graft to generate bone formation [12].

We studied the local effects of alendronate at a previously defined dose [2]. There is no information on how much alendronate remains within the bone grafts at this dose. Studies analysing the effects of different alendronate concentrations and impregnation durations should be conducted to provide further information. From our data, we can conclude that systemic application of alendronate provides improvements in terms of bone density and bone graft integration. Local application at the given dose has stronger effects, but interfered with the infection control process.

Before recommending local bisphosphonates as an adjuvant agent for prevention of osteolysis, bone ingrowth stimulant or bone-targeting device, their possible effects on the immune system should be clarified with future studies.

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