Microstructural properties of bone in rat vertebra after long-term clodronate treatment

Antti Koivukangas¹, Juha Tuukkanen², Petri Lehenkari¹, Raija Peura³, Ritva Hannuniemi⁴, Katriina Kippo⁴, Timo Jämsä⁵, and Pekka Jalovaara¹

¹Division of Orthopedic Surgery, University of Oulu, P.O. Box 5000, FIN-90014 University of Oulu, Finland
²Department of Anatomy and Cell Biology, University of Oulu, Oulu, Finland
³Institute of Electron Optics, University of Oulu, Oulu, Finland
⁴Biomedical Research Center, Leiras Oy, Turku, Finland
⁵Department of Medical Technology, University of Oulu, Oulu, Finland

Abstract Bisphosphonates (BPs) are known to increase bone mineral density, but it is not known how this increase manifests at low hierarchic levels of the bone structure. The present study aimed to clarify the effects of the long-term use of clodronate on the microstructure and chemical composition of bone. The second lumbar vertebral body (L2) in growing rats, subjected to 32 weeks' treatment with clodronate at either a therapeutic dose of 2 mg/kg, or a high dose of 10 mg/kg, or physiological saline (control group), was studied by scanning electron microscopy for morphology, by backscattered electron image (BSE) for density, and by energy dispersive spectrometry for material analysis. BSE images showed that the degree of mineralization in the different areas of trabecular bone of the vertebral body varied in both the control and the study groups, but this variation seemed to be different in the control and study groups. BSE analysis showed that there was more high-density bone (white area) in the low-dose clodronate group than in the controls, but the difference between the high-dose clodronate group and the control group was not significant. The density of the white area (high-density bone) was slightly increased in the low-dose clodronate group. There were no differences in the density of the gray area (low-density bone) between the groups. Neither the distribution of Ca, P, or Mg, nor the total mineral content, was affected by the clodronate treatment. Our results indicate that long-term clodronate treatment at the therapeutic level increases the proportion of high-density bone in the vertebral body in non-osteoporotic rats.

Key words bisphosphonate · clodronate · long-term · rat

Introduction

Bisphosphonates (BPs) decrease bone turnover and, therefore, bone loss [1]. They are widely used in the treatment of bone disorders associated with increased bone resorption, such as osteoporosis, Paget’s disease, and metastatic bone diseases [2,3].

The accumulation of BPs in bone reaches a plateau only after a very long time. In humans, this means years or even decades of administration [1]. It is also known that the skeletal half-life of BPs is long, ranging between 3 months and 1 year [4].

Short-term treatments with therapeutic doses of BPs are generally well tolerated in both experimental and clinical contexts [4,5], whereas etidronate at high doses has been shown to impair normal mineralization in animals as well as in humans [5,6], and impaired mineralization has also been reported after pamidronate treatment of Paget’s disease and fibrous dysplasia [7].

There are only a few experimental studies of the long-term effects of BPs on the skeleton. Etidronate has been shown to provoke spontaneous fractures and impair normal mineralization in dogs after 12 months of treatment [6]. Beneficial bone effects of 2-year alendronate treatment in rats have been reported [8]. Moreover, 1-year tiludronate treatment administered to growing monkeys turned out to be safe [9], and 1-year pamidronate treatment of dogs increased bone stiffness, as calculated sonographically from the dog sternum [10]. In long-term studies of experimental osteoporosis, 2-year treatment of baboons with alendronate [11] and zoledronate treatment of monkeys for 69 weeks prevented experimental osteopenia [12] and involved no adverse effects. There are, however, only a few studies concerning the long-term effects of clodronate in growing rats [13,14]. These studies have shown that long-term treatment has a beneficial effect on bone density and strength at the macroscopic level, while a high dose of clodronate decreased the bone growth rate.

The ultrastructural properties of bone after 4 months of clodronate treatment were studied by Wink as early as 1986 [15]. This study showed that treatment with clodronate prevented the endosteal bone surface
changes that occurred in experimental osteoporosis. Scanning electron microscopy (SEM), alone [16] or with the back-scattered electron (BSE) method, is a powerful tool for determining the microstructure of bone. The effect of tiludronate on bone ultrastructure was studied using this method [17]. It turned out that tiludronate slightly increased the width of bone apatite crystals, but did not affect any other parameters.

The present study aimed to clarify the effects of the long-term use of clodronate on bone at the microstructural level and the effects on the chemical composition of bone, using SEM method with BSE and energy dispersive spectrometry (EDS).

Materials and methods

Animals

A total of 15 female 3-month-old Sprague-Dawley rats, weighing 225 (±12) g, from another study were treated in three different ways for 32 weeks. The animals in group 1 received physiological saline (control), the animals in group 2 were administered 2mg/kg disodium clodronate (low-dose) (Bonefos; Leiras Oy, Turku, Finland), and the animals in group 3 were administered 10mg/kg of disodium clodronate (high-dose). The lower dose (2mg/kg) was chosen as a therapeutic dose, and the higher dose (10mg/kg) was five times higher, for safety purposes. All injections were given subcutaneously (s.c.) twice a week. The subcutaneous administration route was selected because the absorption of clodronate after oral administration is low.

The animals were fed, ad libitum, with a special quality control (SQC) rat and mouse maintenance diet (RM1(E) SQC; Special Diets Services, Witham, England) and allowed free access to tap water. The feed contained 0.71% calcium, 0.50% phosphorus, and 0.60IU/g vitamin D3. Food and water consumption was not determined. The rats were housed in individual cages at a constant temperature (21 ± 1.5°C) and relative humidity (30%–65%), with a 12-h light and darkness cycle (lights on at 7.00 a.m.). The experimental procedures were reviewed and approved by the Ethics Committee of Animal Experimentation in the local Provincial State Office of Western Finland.

At necropsy, the L2 vertebrae were dissected out. The bones were wrapped in saline-soaked gauze and stored in closed tubes at −20°C.

The organic material was removed carefully, and the bones were dehydrated through a graded ethanol series and embedded in polymethyl methacrylate. The bones were cut transversely, across the middle of the L2 vertebra, with a diamond saw, into blocks with a core of central trabecular bone and a cortical shell. The blocks were then ground to a thickness of 100µm, polished, and coated with carbon. All of the analytical methods were applied in a blinded manner.

Scanning electron microscopy (SEM)

SEM analyses were performed with a Jeol JSM-6400 microscope (Jeol, Tokyo, Japan). BSE images were used to evaluate the degree of mineralization. To distinguish between low-density bone (gray areas) and high-density bone (white areas), SEM images were obtained using BSE. In this method, the BSE signal is converted into a digital gray-scale image, where the intensity (gray level) of any pixel in the image is proportional to the mean atomic number of the corresponding location on the target material [18]. The acceleration voltage was 20kV and the working distance, 8mm. BSE images were collected at 2664 × 2000 pixel resolution with 256 gray levels. To ensure the stability of the instruments, the BSE images were calibrated using an aluminum standard. Two BSE images at 200 × magnification were collected from the trabecular bone in the vertebral body.

Image analysis

The BSE images were analyzed using a digital image analysis system (MCID/M4 with software version 3.0 rev. 1.1; Imaging Research, St. Catharines, Canada). The threshold between the gray and white areas in each image was chosen to distinguish the histological differences optimally [19]. The proportions of the gray and white areas were quantified. The absolute value for image optical density (IOD) was calculated from ten randomly selected spots in the gray area and ten spots in the white area in both images of each bone. The mean width of interstitial trabecular osteons (OsWi) was calculated from ten osteons in both images of each bone.

Energy dispersive spectrometry (EDS)

The concentrations (wt %) of Ca, P, and Mg and the total mineral content in each sample were quantified using a scanning electron microscope equipped with EDS (INCA 3.03; Oxford Instruments, Witney, UK). The SEM operating conditions were 15kV acceleration voltage and a working distance of 15mm. Analyses were performed at ten points in the gray areas and at ten points in the white areas in each bone.

Statistical analysis

The average value of the ten measurements of each image was used in the statistical analyses. Pairwise group comparisons were done with the nonparametric