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Doses effects of zoledronic acid on mineral apatite and collagen quality of newly-formed bone in the rat's calvaria defect

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ABSTRACT

Due to their inhibitory effects on resorption, bisphosphonates are widely used in the treatment of diseases associated to an extensive bone loss. Yet, little is known about bisphosphonates effects on newly-formed bone quality. In the present study, adult male Sprague-Dawley rats (n = 80) with a bone defect *calvaria* area were used and short-term effects of zoledronic acid (ZA) were studied on the healing bone area. Three ZA treatments were tested by using either: 1°) a low single dose (120 μg ZA/kg, n = 10; equivalent to human osteoporosis treatment), 2°) a low fractionated doses (20 μg ZA/kg daily for 6 days either a total of 120 μg/kg, n = 15), and 3°) a high fractionated doses, (100 μg ZA/kg weekly for 6 weeks, n = 15; equivalent to 6 months of human bone metastasis treatment). For each treatment, a control "vehicle" treatment was performed (with an identical number of rats). After ZA administration, the intrinsic bone material properties were evaluated by quantitative backscattered electron imaging (qBEI) and Raman microspectroscopy. Neither single nor fractionated low ZA doses modify the intrinsic bone material properties of the newly-formed bone compared to their respective control animals. On the opposite, the high ZA treatment resulted in a significant decrease of the crystallinity (−25%, $P < 0.05$) and of the hydroxyproline-to-proline ratio (−30%, $P < 0.05$) in newly-formed bones. Moreover, with the high ZA treatment, the crystallinity was positively correlated with the hydroxyproline-to-proline ratio ($\rho = 0.78$, $P < 0.0001$). The present data highlight new properties for ZA on bone formation in a craniofacial defect model. As such, ZA at high doses disrupted the apatite crystal organization. In addition, we report here for the first time that high ZA doses decreased the hydroxyproline-to-proline ratio suggesting that ZA may affect the early collagen organization during the bone healing.

1. Introduction

Bisphosphonates (BPs) are known for their antiresorptive effects and their clinical properties related to the improvement of bone strength. BPs also show strong affinity for bone mineral, and their benefits are thought to be through their selective accumulation and improvement in bone quality, particularly in intrinsic bone material properties [1]. These effects were shown in bones going under remodeling with the increased organic and mineral bone tissue maturities. Yet, the remodeling suppression of BPs is only partial and some new bone matrix can be formed under treatment [2]. The newly-formed bone can be targeted by BPs because of the availability of the newly-formed mineral crystals for binding during BPs uptake and/or BPs recycling [3]. In addition, the potential adverse effects or interferences of BPs treatments on bone formation have been previously questioned in bone modeling [4–6], long bones fracture healing repair [7–9], *maxillae* bone healing [10–13] and osseointegration processes [14–16]. How BPs could affect the bone material properties in newly-formed bones remains unclear.

Zoledronic acid (ZA), a potent third-generation amino-BP, is currently known to have a higher mineral binding affinity and a higher antiresorptive activity than other BPs [17]. On actively forming bone surfaces, ZA has been previously shown to reverse deleterious postmenopausal osteoporotic effects as well as to exert also positive effects on bone quality on the younger bones [18,19]. This enhancement of the bone quality by ZA was suggested to be independent from its antiresorptive effects, possibly by improving directly bone formation and maturation. Nevertheless, the influence of ZA on bone matrix formation and mineralization needs to be explored.

The rodent *calvarial* bone defect is widely used to evaluate bone healing processes [20–22] or effects of various local factors on bone

Abbreviations: BE, back-scattered electrons; BMDD, bone mineralization density distribution; BP, bisphosphonate; CTL, control; GAG, glycosaminoglycan; PMMA, polymethyl methacrylate; qBEI, quantitative backscattered electron imaging; ROI, region of interest; ZA, zoledronic acid.

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formation [23–25]. This craniofacial bone site in rodent represents a suitable alternative model to evaluate bone formation without mechanical loading of the bone due to its localization [26]. We hypothesized that BPs treatment modify the mineral and organic components of the newly-formed bone in craniofacial bone region. The aim of the study was thus to determine the ZA effects on bone material properties within the repaired bone defect area in the rat model. We proposed to examine the alterations of the newly-formed bone by Raman microspectroscopy and quantitative backscattered electron imaging (qBEI). In particular, we studied the relative gain/loss of newly-formed bone in ZA-treated rats with low ZA doses (i.e., those used for treating osteoporosis) or high ZA doses (i.e., those used for treating malignant bone diseases).

2. Materials and methods

2.1. Animals

Eighty adult male Sprague Dawley rats aged 56-day-old (400 g ± 20 g) were purchased from Elevage Janvier (Le Genest-Saint-Isles, France). All animals were housed individually in light- and temperature-controlled facilities and maintain with ad libitum access to a standard laboratory diet and water. All animals and surgical procedures were approved by the Veterinary Department of the French Ministry of Agriculture (approval no. 59-350137). The animals were treated in accordance with the guidelines of the Hospitalo-Universitary Department of Experimental Research of Lille (DHURE) and the Guide for Care and Use of Laboratory Animals (NIH publication 93-23).

2.2. Surgical procedure

Surgical procedures were performed under ketamine (50 mg/kg, Imalgen®) and xylasin (5 mg/kg, Rompun®). A dose of carprofen (15 mg/kg, Rimaldyl®) was administrated to control postoperative pain. After aseptic preparation, a semilunar incision was made through the skin and periosteum at the top of the *calvarium*, allowing reflection of a full thickness flap in posterior direction. A custom-made surgical guide was placed on the bone and fixed using two micro screws (0.9 × 1.3 mm Modus®, Mediartis S.A.R.L., Vaulx Milieu, France). A 4-mm diameter defect was made on sagittal suture with a trephine (Stoma®, Emmingen-Liptingen, Germany) using a low-speed handpiece under sterile saline irrigation. The surgical guide was removed and the micro screws were left as references to allow localization of the original margins of the surgical defect after bone healing. The soft tissues were then repositioned and sutured to achieve primary closure (4-0 threads Vicryl Ethicon®, Norderstedt, Germany). All animals exhibited normal activity and showed an increase in weight after surgery.

2.3. Dosing regimens

Zoledronic acid (ZA; 2-(imidazole-1-yl)-hydroxy-ethylidene-1, 1-bisphosphonic acid, disodium salt, 4.75 hydrate) was provided by Novartis Pharma (Basel, Switzerland). The drug was first dissolved in sterile physiological saline (0.9% NaCl) at a concentration of 50 μg/mL and then further diluted to the given concentration to be administrated for each animal.

Two weeks after surgery, animals were randomly treated with subcutaneous dose of vehicle (sterile physiological saline, 0.9%) or ZA following the same protocol. The randomization schedule was generated by an online statistical computing web programming (www.randomization.com).

Three doses of ZA were used as follows (Fig. 1):

- group ZA-S120 (n = 10): one single dose of 120 μg/kg body weight (equivalent to human osteoporosis treatment [27]);

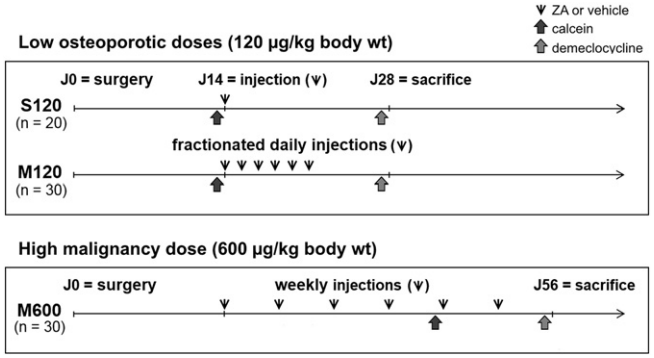

Fig. 1. Time-line diagram of injections procedures according the dosing regimen (low doses of 120 μg/kg body wt. or high dose of 600 μg/kg body wt.) and the administration rhythm (single, S or multiple, M injections). Two weeks after surgery, among each group S120, M120 and M600, an equivalent number of animals received randomly zoledronic acid (ZA) or vehicle. Additionally, animals received injections of 30 mg/kg body wt. calcein and demeclocycline at 15 days and 1 day before sacrifice respectively.

- group ZA-M120 (n = 15): a daily dose of 20 μg/kg body weight for 6 days (either the fractionated of 120 μg/kg dosage);
- group ZA-M600 (n = 15): one weekly dose of 100 μg/kg body weight for 6 weeks (equivalent to 6 months of human bone metastasis treatment [28,29]).

Similarly, a same number of animals were treated with vehicle to provide the respective control groups: CTL-S120 (n = 10), CTL-M120 (n = 15) and CTL-M600 (n = 15) groups.

To identify the newly-formed bone area, a double labelling was performed for which animals received intraperitoneally injections of calcein and demeclocycline (30 mg/kg body weight, Sigma-Aldrich®, Saint-Quentin Fallavier, France) at 15 days and 1 day before sacrifice respectively.

2.4. Tissue processing

At 4 weeks post-operation (groups S120 and M120) or 8 weeks post-operation (group M600), animals were sacrificed by lethal intracardiac injection of T61 under general sedation. The complete *calvarium* area was harvested and fixed in 70% ethanol solution for 48 h. Samples were embedded in poly-methyl methacrylate (PMMA). A 100-μm-thick section were cut in the cranio-caudal plane and progressively polished with silicium carbides and diamond suspension (Escil®, Chavassieu, France).

2.5. Selection of the bone areas

Imaging of fluorescence labelling was performed with an epifluorescence confocal microscope Zeiss LSM710 (Carl Zeiss, France) and the associated Zen 2009 software. The microscope was equipped with an Apochromat objective (×40, NA = 1.2), a multiline argon laser, and adequate filter blocks. For each sample, the acquisition of the entire *calvarium* bone section was performed to identify the newly-formed bone (defect bone area) from the native bone (distant bone areas). In the defect bone area, three distinct sampling areas were selected between the two fluorescent labelling (Fig. 2A). In some cases (3/80 animals), the signal of demeclocycline labelling was very low. In these cases, for which only calcein label was clearly discernible, the sampling areas were chosen in the calcein labelling area because we previously demonstrated that fluorescence did not affect the bone quality parameters [30]. In the distant area, three distinct sampling areas without fluorescent labelling were chosen to achieve comparison (Fig. 2B). For each sample, the quantitative backscattered electron