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Search for a reliable model for bisphosphonate-related osteonecrosis of the jaw: establishment of a model in pigs and description of its histomorphometric characteristics

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Abstract

The pathogenesis of bisphosphonate-related osteonecrosis of the jaw (BRONJ) remains unknown, and the development of a reliable experimental model would help to improve our understanding of it. We used 12 domestic pigs of which 6 made up the experimental group and were treated with zoledronate 4 mg intravenously weekly for 5 weeks, while the control group (n = 6) were given no drugs. On day 60 the right second maxillary and mandibular third molars were extracted. Thirty days later 3 animals in each group were killed; the rest were killed 90 days later. Histopathological specimens from the extraction sites were analysed for bone density, collagen architecture, density of osteons, and the amount of non-mineralised bone. Bone density, amount of non-mineralised bone, and density of osteons differed significantly between the 2 groups ($p < 0.001$ in each case), but the chromatic pattern dictated by the collagen architecture did not. Our results correspond to the observations that have been made in patients with BRONJ, which means that the histomorphometric conditions seen in patients can be reproduced in this experimental setting.
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Keywords: Bisphosphonates; Bisphosphonate-Related Osteonecrosis of the Jaws; Experimental model

Introduction

Bisphosphonates are used mainly for malignancy-related skeletal problems such as osseous metastases, hypercalcaemia, and multiple myeloma, as well as osteoporosis. Their antiresorptive effect on bone is achieved through the suppression of activation of osteoclasts and the induction of their apoptosis.[1–4]

An important side effect of bisphosphonates is bisphosphonate-related osteonecrosis of the jaw (BRONJ), which has become an established clinical diagnosis.[1–4] The most widely-accepted diagnostic criteria were proposed by the American Association of Oral and Maxillofacial Surgeons in 2009 and maintained in the 2014 revision of the relevant guidelines, and are: current or previous treatment with a bisphosphonate; exposed bone in the maxillofacial region that has persisted for more than 8 weeks; and no history of radiotherapy to the jaws.[2]

The aetiology and pathogenesis are unresolved,[1–6] as no theory has yet been able to fully explain them,[5,6] and there is no internationally accepted protocol for management.[3] Treatments range from medical management to minimally invasive

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surgery to major surgery.[3] Development of a reliable experimental model would be useful to increase our understanding of BRONJ and test various treatment protocols.

Here we describe an experimental large animal model (domestic pigs) that aims to reproduce some of the osseous characteristics seen in humans who are treated with bisphosphonates, after a surgical trauma to the jaws.

Some important osseous characteristics in patients with BRONJ include: reduced osteon density compared with normal tissue;[6–9] increased apposition of woven bone between the osteons[6–9] (woven bone is known to mineralise quicker than lamellar bone,[10] so in a region where bone heals, an increased ratio of mineralised: non-mineralised bone would be expected in cases of BRONJ); hyperdensity in the radiographic examination[11] caused by the increased apposition of woven bone and mineralisation; and no changes in the collagen content of the bone, as BRONJ is not a fibroatrophic process like other types of osteonecrosis such as osteoradionecrosis.[6]

For our model to be accurate and useful these characteristics should be reproduced.

Our null-hypotheses therefore were: there is no significant difference between the control and the experimental group in osteon density; the ratio of mineralised:non-mineralised bone does not differ significantly between the control and the experimental groups; there is no significant difference in the radiologically-assessed bone density between the control and the experimental groups; nor is there a significant difference between the collagen content in the 2 groups.

Material and methods

Experimental setting

Domestic pigs were used for the experiment under license (L.N.22.1/121/3/2011), and were treated according to the EU Directive 2010/63/EU for animal experiments. Animals weighed about 100 kg each on delivery and were kept at a temperature of 18 °C with the normal circadian rhythm preserved. Each animal had a living area of 6 m^2 covered with straw, and they were fed standard food. They had free access to water and their wellbeing was monitored by a vet.

Twelve animals were used, divided into 2 groups of 6. The 6 experimental animals were given zoledronate (Novartis, Switzerland) 4 mg intravenously (the highest dose given to humans) once a week for 5 weeks. Those in the control group were given no drugs. The zoledronate was given under sedation with midazolam 1 mg/kg and ketamine (10 mg/kg).

On day 60 all animals had the right second maxillary and mandibular molars extracted under general anaesthesia induced by midazolam (1 mg/kg) and ketamine (10 mg/kg). To minimise the risk of infection a perioperative dose of penicillin or streptomycin 15 mg/kg was given. Buprenorphine 0.1 mg/kg was given subcutaneously twice daily to control pain.

Thirty days after extraction three of the animals in each group were killed, the others being killed after a further 60 days. They were sedated with ketamine (10 mg/kg) and midazolam (1 mg/kg) given intramuscularly, and then given 20% pentobarbital solution intravenously. The skulls and mandibles were removed for further analyses.

Preparation of histological slides

Histopathological specimens were obtained from the extraction sites, and cut in fine layers using the method described by Donath and Breuner.[12] After being fixed with a cyanoacrylate (Technovit® 7210 VLC, Heraeus-Kulzer, Wehrheim, Germany) they were cut into slides 300 μm thick.

Radiographic assessment of the newly formed bone

The specimens were then cut into sections 90 μm thick. Radiographs were taken in a Faxitron® cabinet (Faxitron, Tuscon, USA; Rohde and Schwarz, Germany) for 6 minutes at 13 kV and 2.5 mA, and the radiographs digitised using a scanner at 2400 dpi and 8-bit grey scale, and evaluated with Bioquant-Osteo V7.10.10 (Bioquant Image Analysis Corporation, Nashville, USA). The density of the bone was measured as a percentage.

Histomorphometry

The specimens were stained with toluidine blue and viewed using a Zeiss light microscope (Axioskop, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Images were digitised and evaluated using the Bioquant-Osteo software. The osteon density and the ratio of mineralised:non-mineralised bone were examined.

The collagen architecture, and more specifically, the relative presence of collagen I and III, was evaluated under polarised light as described by Mitsimponas et al[6] after the specimens had been stained with Sirius Red. The chromatic pattern displayed by the specimens was assessed by RGB (red-green-blue) analysis and, more specifically, by looking at the ratios of the intensity of each of the basic colours to the brightness of the image (RGB value).

Statistical analysis

Statistical analyses were done with the help of SPSS software version 21 (IBM Corp, Armonk, USA). Probabilities of less than 0.05 were accepted as significant (to accept or reject the null-hypotheses). We used Student's *t* test (to compare 2 groups) and analysis of variance (ANOVA, for more than 2), and when a probability was given as less than 0.05 we used post-hoc tests. To strengthen our analysis, we also used the Kruskal-Wallis non-parametric test for more than 2 groups.

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