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Introducing a protocol to create bisphosphonate-related osteonecrosis of the jaw in rat animal model

Mohammad Zandi [a, b], Arash Dehghan [c], Hamid Malekzadeh [a, *], Pejman Janbaz [a], Khaled Ghadermazi [a], Payam Amini [d]

[a] Department of Oral and Maxillofacial Surgery, Hamedan University of Medical Sciences, Hamedan, Iran
[b] Dental Research Center, Hamedan University of Medical Sciences, Hamedan, Iran
[c] Department of Pathology, Hamedan University of Medical Sciences, Hamedan, Iran
[d] Department of Biostatistics, Hamedan University of Medical Sciences, Hamedan, Iran

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ABSTRACT

Objective: Previously published animal investigations on bisphosphonate-related osteonecrosis of the jaws (BRONJ) showed a variety of methods for BRONJ induction and inconsistent findings. The aim of present study was to develop a reliable protocol for BRONJ induction in rat animal model.
Subjects and methods: In a pilot study, 64 rats were randomly divided into 4 groups and 16 subgroups (each containing 2 experimental and 2 control rats) based on the timing of tooth extraction and euthanasia. The experimental and control rats received intraperitoneal injection of 0.06 mg/kg zoledronate and saline, respectively, once a week until sacrificed, and evaluated for presence of bone exposure clinically, and osteonecrosis and new bone formation histologically. The protocol that successfully produced BRONJ in pilot study was tested in a randomized controlled experimental investigation using 45 rats.
Results: In pilot investigation, the highest rate of BRONJ was obtained after four weekly zoledronate injections, at least 4 weeks after tooth extraction. The randomized controlled experimental study verified this finding with a success rate of 83%, and also showed that more prolongation of zoledronate therapy did not increase the BRONJ rate.
Conclusion: The protocol developed in the present study could be used reliably for future BRONJ investigations on rats.

1. Introduction

Bisphosphonates (BPs) are a class of pharmaceutical agents that bind to hydroxyapatite of bone and inhibit osteoclastic activity and bone loss. So, they are widely used in the treatment of osteoporosis, multiple myeloma, bone metastases from solid tumours, and other conditions that involve bone resorption (Lewiecki, 2011). A serious complication reported in patients receiving nitrogen-containing BPs is necrosis of the jaw bones. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is characterized by exposed bone or bone that can be probed through an intraoral or extraoral fistula in the maxillofacial region, lasting for more than eight weeks, caused by oral or intravenous bisphosphonate therapy in patients with no previous radiation therapy or obvious metastatic disease to the jaws. Because of the growing number of osteonecrosis cases involving the jaws associated with other antiresorptive and anti-angiogenic treatments, the term medication-related osteonecrosis of the jaw (MRONJ) was introduced (Ruggiero et al., 2014). Since its first description in 2003 (Marx, 2003), an increasing number of cases with BRONJ have been reported in the literature. Most of the reported cases occurred after tooth extraction, dentoalveolar surgery or denture trauma, and several cases spontaneously (Kos et al., 2010; Otto et al., 2012; Pichardo and van Merkesteyn, 2013; Vaszilko et al., 2014). According to previous studies, most cases of BRONJ were associated with intravenous forms of BPs (2007; Dodson, 2009; Fleisher et al., 2013).

Although over a decade has passed since initial presentation of BRONJ, its pathogenesis is still controversial, and no standard

* Corresponding author. Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Hamedan University of medical sciences, Shahid Fahmideh street, Hamedan, Iran. Tel: +98 8138381086, +98 9173065089(mobile); fax: +98 8138354220.
E-mail address: hamid_malekzadeh_28@yahoo.com (H. Malekzadeh).

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for prevention and treatment of this condition has yet been clarified (Voss et al., 2012; Vercruysse et al., 2014; Bodem et al., 2015; Otto et al., 2015). The current knowledge about BRONJ is mostly based on case reports and series and retrospective studies published in the literature rather than evidence-based research. Because of potential risk of BRONJ investigations for human health and the low incidence rate of this disease, human experimentations are often unfeasible and unethical. In these cases, animal models could be used successfully to investigate various aspect of the BRONJ disease such as underlying etiopathology and associated risk factors, and prevention and treatment strategies.

Rat is an animal model that is widely used in medical research because it is very common, inexpensive, and easy to breed and maintain. Several BRONJ investigations have been previously performed on rats but their results were inconsistent. In these studies, different protocols (the type, dose, interval, duration, and route of administration of the BP medication, and the use of co-medications) for BRONJ induction had been used. Furthermore, in most of the previous studies, the diagnosis of BRONJ was based on histopathological not clinical findings, and the persistence of bone exposure and/or fistula for at least 8 weeks (a criterion currently required for diagnosis of BRONJ) was not followed (Abtahi et al., 2013; Barba-Recreo et al., 2014; Dayisoylu et al., 2014; Sakaguchi et al., 2015). For the findings of various animal studies to be reliable and comparable, development of a standard and universally accepted protocol for BRONJ induction in rats is required.

The aim of the present study was to develop a reliable and reproducible protocol for BRONJ induction in a rat animal model.

2. Material and methods

The protocol for this research was reviewed and approved by the Hamedan University of Medical Sciences Ethics Committee.

The present investigation consisted of a pilot investigation and a randomized, controlled, prospective experimental study.

In the pilot study, 64 Wistar Albino rats with a mean age of 10 weeks and weight of 300–350 g were involved. The animals were obtained from the Animal House of the University and allowed to acclimatize to the laboratory condition for 10 days before being used. The rats were randomly distributed into 4 groups of A to D (each 16 rats) according to the timing of tooth extraction, and each group was further divided into 4 subgroups (A1 to A4, B1 to B4, C1 to C4, and D1 to D4), according to the timing of euthanasia. Of four animals in each subgroup, two served as experimental and two as control rats. The experimental rats (n = 32) were administered intraperitoneal injection of 0.06 mg/kg zoledronate (Zometa, Novartis Pharma, Basel, Switzerland) and the control rats (n = 32), the same volume of saline solution, once a week (first injection at the starting day of study) until sacrificed.

All of the 64 rats were subjected to bilateral mandibular tooth extraction under intraperitoneal general anaesthesia using 75 mg/kg of Ketamine hydrochloride (Rotexmedica, Trittau, Germany) and 7.5 mg/kg of midazolam (Midazolex, Exir, Iran). Tooth extraction for animals in groups A to D was performed coincident with 1st to 4th intraperitoneal zoledronate/saline injection, respectively. After placing the rat in a supine position, the gingiva around right and left mandibular first molars was detached, and the teeth were extracted using a sharp dental explorer. Using an overdose of anaesthetic, the animals in subgroups A1 to A4 were euthanized 1–4 weeks after tooth extraction, respectively. The same time schedule for euthanasia was followed for the rats in groups B to D. The outline of the pilot study design is summarized in Fig. 1.

2.1. Macroscopic evaluation

Following euthanasia, the rats were clinically examined for presence of any bone exposure and intraoral or extraoral fistula.

2.2. Histological analysis

The mandibles of 64 sacrificed rats were excised. The 128 (64 experimental and 64 control) hemimandibles fixed in 10% formalin solution, decalcified with EDTA, and embedded in paraffin. The samples were sectioned (serial 4 μm slices) buccolingually at the tooth extraction area, parallel to the long axis of the adjacent teeth, and stained with haematoxylin and eosin. In histological examination, the presence of necrotic bone around extraction site and new bone formation in the socket were evaluated. In present study, the presence of 8 contiguous empty lacunae (no osteocyte) in the bone adjacent to extraction socket was considered as bone necrosis.

After completion of the pilot study, one of the protocols that produced BRONJ with a high success rate (subgroup D4) was tested in a randomized, controlled experimental investigation using a larger sample size and with a longer period of follow-up. In this experimental study, it was also investigated if increasing the duration of BPs therapy longer than the period provided in pilot study would increase the incidence rate of BRONJ in rat model. A total of 45 rats were randomly divided into 3 groups: two experimental groups of S1 and S2 (15 rats in each), and one control group (15 rats). The rats in groups S1 and S2 received intraperitoneal injection of 0.06 mg/kg of zoledronate, and the control rats the same volume of saline solution, once a week (first injection at the starting day of study) until sacrificed. Bilateral mandibular tooth extraction was performed at day 21 of the experiment for rats in groups S1 and control, which was in accordance with the pilot study protocol performed for subgroup D4. For rats in group S2, tooth extraction was conducted at the end of the week 6 (3 weeks later). The bone exposure and/or fistula were observed for 8 weeks (to be in accordance with current definition of BRONJ) and then the rats were sacrificed. The 90 hemimandibles were subjected to the same clinical and histological examinations as the pilot study.

In the present study, the examiner who clinically evaluated the rats and also the pathologist were blind to group/subgroup assignment of animals.

For statistical analysis of the data obtained in the present study, SPSS 16.0 (SPSS Inc., Chicago, IL) software was used. Pairwise intergroup comparisons were performed by Pearson's chi-squared and Fisher's exact tests, and $P < 0.05$ was considered statistically significant.

3. Results

The experiment was well tolerated by all rats. The longer the duration of zoledronate administration, the more weight loss was observed in experimental rats.

3.1. Pilot study

In the pilot investigation, a total of 128 hemimandibles were examined for the presence of bone exposure and/or fistula, bone necrosis, and new bone formation. The clinical and histological findings are presented in Tables 1–3.

3.1.1. Bone exposure and/or fistula

In groups A to D, one week after tooth extraction, no soft tissue healing was observed in both experimental and control rats. Two weeks after tooth extraction, soft tissue healing occurred in all except one of the 16 control hemimandibles. However, 12 of 16