Chemical Features of Bisphosphonates and Histopathological Outcomes in Rat Jaw Treated with Zoledronic Acid Combined with Dexamethasone

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Bisphosphonate related osteonecrosis of the jaws (BRONJ) is a pathological entity described for the first time in 2003; three criteria are mandatory: no radiotion therapy, exposed bone in oral cavities for at least eight weeks with no signs of healing, bisphosphonate therapy in present or in the past, in patients with bisphosphonate therapy in present or in the past. Bisphosphonates (BPs) are stable analogues of natural inorganic pyrophosphate, which inhibit bone resorption. In literature, most patients diagnosed with BRONJ were suffering from multiple myeloma and they had a treatment with nitrogen containing BPs and steroids such as dexamethasone intravenously. With this study we have aimed to achieve a rat model of BRONJ and to evaluate histopathological findings how concurrent use of BPs and steroids can affect emergence of BRONJ in this study.

Keywords: bisphosphonates, dexamethasone, osteonecrosis, jaw, zoledronic acid

Bisphosphonate related osteonecrosis of the jaws (BRONJ) was described for the first time as a pathological entity by Marx in 2003 when he presented 36 cases of BRONJ in patients suffering from malignant tumors in the USA [1].

In 2009 the American Association of Oral and Maxillofacial Surgeons (AAOMS) established BRONJ as exposed bone in oral cavities, in patients who have been taken bisphosphonates, they had no radiation therapy and the exposed bone persisted at least eight weeks with no signs of healing [2,32,33].

The Australian and New Zealand Bone and Mineral Society, Medical Oncology Group of Australia, Osteoporosis Australia and the Australian Dental Association define BRONJ as an exposed portions of bone at least for six weeks [3,34,35].

In September 2013 the AAOMS committee was reconvened to debate current literature and guidelines for diagnosis and treatment of this pathology. This special committee has changed its name and prefers the term medication-related osteonecrosis of the jaw (MRONJ) as a result of increases in cases of jaw osteonecrosis consecutive to therapy with other antiresorptive drugs, such as denosumab, or antiangiogenic medication. To diagnose MRONJ, they propose to meet three criteria, namely: therapy with antiresorptive or antiangiogenic drugs currently or in the past, for at least eight weeks exposed bone in the oral cavity without signs of healing, and the patient was not exposed to radiation treatment in the past [4,36-38].

BRÓNJ is related to type of BPs, dose, and route administration. BPs containing nitrogen or intravenously administration is more likely to cause BRONJ compared with BPs without nitrogen or administered orally. Alveolar surgical trauma, infection inflammation in the oral cavity and chemotherapeutic combination with immunosuppressive agents such as dexamethasone, also have an increased risk of inducing osteonecrosis of the jaw [2,39,40].

Bisphosphonates ((HO)2P(O)CR1R2P(O)(OH)2) are stable analogues of natural inorganic pyrophosphate, conferred on the strenght of a carbon atom replacing the oxygen atom that connects the two phosphates. These agents have the ability to chelate calcium ions, target rapidly to bone mineral [5,41]. Chemically, BPs of medical interest are characterized by two phosphonate groups sharing a common carbon atom (P-C-P) resistant to enzymatic hydrolysis. These leads to excreting BPs unaltareted.

BPs, which inhibits bone resorption, are extensively used to treat osteoporosis, bone metastases of malignant tumors (multiple myeloma, breast cancer, prostatic cancer), and Paget's disease of bone [6-8]. By increasing strength and bone mineral density and decreasing the risk of bone fractures, BPs significantly improve the quality of life.

In literature, most of the patients who were diagnosed with BRONJ were suffering from multiple myeloma, breast cancer, and prostate cancer. Also, these patients had a treatment with nitrogen containing BPs and steroids such as dexamethasone intravenously. However, it was not seen a connection between systemic risk factors, competing drugs use and the appearance and worsening of BRONJ. BPs type, route of administration, patient age associated with periodontal disease, tooth caries decay implications and their treatment (tooth extraction, dento-alveolar surgery), concomitant use of drugs are associated risk factors of BRONJ [9,10]. Of these, tooth extraction and dento-alveolar surgery are the most significant factors [9].

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Research conducted to date has not identified a direct cause for BRONJ in vivo. It is still necessary to demonstrate that intravenous regimens of BPs consistently induce BRONJ in animals, regardless of pre-existing comorbidities or local infection. In updating their position in 2014, the AAMOS stressed the need for reliable animal models that can be used to test possible prevention and treatment protocols [4]. We have aimed to achieve a rat model of BRONJ and to confirm how concurrent use of BPs and steroids can affect emergence of BRONJ in this study.

Experimental part

Animals Groups

The experimental procedures were reviewed and performed by the guidelines of the Institutional Animal Care and Ethical Committees, at University of Medicine and Pharmacy Targu Mures (Protocol number 79, Date: 09/22/ 2014).

Wistar male rats aged 10–12 weeks and weighing between 450 g and 550 g were used. Animals were randomly assigned into three groups of twelve animals each. Animals were housed in labeled cages and observed before study start. Animals were fed with a standard rat food and watered *ad libidum* for the duration of the experiments. A 12 h light/dark cycle was maintained with a filtered air at a temperature of 22 ± 0.6 Celcius degrees and $50 \pm 20\%$ relative humidity.

Study design

Thirty-six (36) rats were prospectively and randomly divided into three groups of twelve (12) animals each. Local surgical trauma were performed seven days following the final dose of one of three regimens consisting of zoledronic acid (ZA) and dexamethasone (DX) subcutaneously administered in group 1, zoledronic acid (ZA) subcutaneously administered at group 2 and saline solution subcutaneously administered in group 3 (Table 1). Local surgical trauma were performed in the palatal area nearby right maxillary molars.

The doses have been established based on doses administered to humans.

Before local surgical trauma, animals were anesthetized with a single intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine (5 mg/kg). Local anesthesia consisting of 2% lidocaine containing 1:100,000 epinephrine was infiltrated into the trauma site as needed. Local trauma was performed in the upper jaw palatal area nearby the last two molars, by experienced operators using a dental drill similar to dento-alveolar surgery procedures, and then the animals received buprenorphine for pain 0.15 mg/ kg intramuscularly three doses in three days.

Euthanasia and Sample Collection

In each group, animals were euthanized after two weeks following the surgical treatment applied. Euthanasia started with the same anesthesia used for the surgical treatment, followed by exsanguination. Blood was withdrawn for serum analysis via an intracardiac catheter. The animals were dissected to collect the upper jaw. The jaws were split in the space between first and second upper molars, followed by the trimming of excess soft tissue. The remaining bones and soft tissue were fixed in 10% formalin. After 72 h they were decalcified in a 10% ethylenediaminetetraacetic acid (EDTA) decalcifying solution for 20 h before being grossly dissected, paraffin embedded, cut into 5 μ m thickness sections, and stained with hematoxylin and eosin (H&E).

Study endpoints

A board-certified oral pathologist performed the histopathological examination using magnifications of 4X, 10X, 20X and evaluate changes in bone vascularity; osteoblastic and osteoclastic activity, empty lacunae of osteoclasts, necrotic bone, the integrity of the overlying epithelium.

Results and discussions

Generally, animals tolerated the procedures well and proved rapid recovery from anesthesia. Four rats from group 2 died during the experiment; we can not identify the cause.

Macroscopic results

In group ZA+DX open wounds were noted in the majority of subjects, and five showed exposed bone. In group ZA open wounds and exposed bone were observed in the majority of all eight rats remained in the experiment, and in group number 3 all subjects proved a real healing (Table 2).

Normal healing occurred in control group: no clinical evidence of BRONJ, no bone exposure, fistula, and mucosal swelling, no sequestra or bone infection was observed in the soft tissue defect sites.

Group number	Group information	Doses and medication	Doses frequency	Local trauma	Euthanasia sample collection			
1(n =12)	ZA+ DX	0.06 ml ZA+ 0.25 ml DX	day 1, day 8, day 15, day 22	day 29	day 43			
2(n =12)	ZA	0.06 ml ZA	day 1, day 8, day 15, day 22	day 29	Day 43			
3(n =12)	control	0.06 ml saline solution	day 1, day 8, day 15, day 22	day 29	Day 43			

Table 1
WE USED THREE GROUPS OF TWELVE ANIMALS EACH

Macroscopic results	Group 1	Group 2	Group 3
Exposed bone	5	7	0
Open wounds	8	7	0
Good healing	0	0	12

 Table 2

 MACROSCOPIC RESULTS

Microscopic results

HE staining was performed to determine: condition of osteoblasts, osteoclasts, presence of exposed bone, inflammatory cell infiltrate and vascularity.

In control group, results showed normal healing of bone, almost entirely healed epithelium. The control group exhibited normal bone remodeling, active osteoblasts and a large number of osteoclasts. Organized connective tissue was present. The samples were free from necrotic bone or inflammatory infiltration. Bone cellularity was normal, rare sequestrations were noted, and the vascularity was unremarkable, no histopathological finding in favor of osteonecrosis was seen (fig. 1).



Fig.1. Histopathological findings of the control group at the soft tissue defect site: A. HE, 10X. Normal bone remodeling, tooth in the right area and squamous epithelium in the inferior area. B. HE, 4X. The defects healed normally, with no microscopic evidence of BRONJ. C. HE, 4X. Bone structure without modifications from normal bone structure. The tooth is also seen on this histopathological section. D. HE, 4X. Normal bone structure, squamous epithelium in the inferior area

In group ZA+DX:

Specimens from animals treated with ZA+DX demonstrated qualitative differences from controls related to the integrity of overlying mucosa, a number of bony sequestra, the extent of bone cellularity. Although focal necrosis was present in many animals treated with ZA+DX, was observed the presence of extensive osteocartilaginous regeneration through chondrocyte hyperplasia in place of bone tissue (fig. 2a, 2b).



Fig.2a. Histopathological findings in the group of animals treated with ZA and DX: A. HE, 4X. Good bone regeneration and fibrous tissue with poorly inflamation. B. HE, 4X. Optimal bone regeneration and minimal necrosis. C. HE, 20X.
Osteocartilaginous regeneration. There is a hyperplasia of chondrocytes in place of bone tissue. D. HE, 20X.
Osteocartilaginous regeneration and necrosis



Fig.2b. Histopathological findings in the group of animals treated with ZA+DX: A. HE, 10X. Osteocartilaginous regeneration. B. HE, 20X. Osteocartilaginous regeneration. C. HE, 10X. Mature osteocartilaginous regeneration. D. HE, 4X. Mature osteocartilaginous regeneration

In group ZA :

In most samples, we could see the appearance of a dense fibrous connective tissue at the level of the created bone defect. Many empty bone lacunae, marginal bone loss, a lot of necrotic bones infiltrated with inflammatory cells (fig. 3a, 3b).



Fig.3a. Histopathological findings in the group of animals treated with ZA: A. HE 10X: Young fibrous tissue in the bone defect. B. HE. 2X: A bone lacunae filled with fibrous tissue.

C. HE, 20X: Detached osteoclasts. D. HE, 10X: Fibrous tissue among bone lamellae



Fig.3b: Histopathological findings in the group of animals treated with ZA: A. HE, 10X: Detached bone lamellae, fibrosis and inflammatory infiltration. B. HE, 4X: Bone lacunae without signs of healing. C. HE, 4X: Bone lacunae with necrosis. D. HE, 10X: Bone and fibrosis with a discreet inflammatory infiltration.

Healing occurs through fibrosis without bone lashes.

HE staining findings of the jaw also suggested the change of osteoblasts induced by zoledro-nate; the test group showed fewer osteoclasts than the control group.

The aim of the current study was to achieve a rat BRONJ model and to confirm how concurrent use of BPs and steroids can affect emergence of BRONJ. To benefit other researchers for this animal model, we achieved a shorter period for BRONJ development in rats, and the exposure to ZA was enough to obtain changes in our study, such as necrosis and fibrosis, present in the majority of ZA-treated rats. This could be explained by differences in bony metabolism and aging rates between rats and humans [11]. In order to compare with the litersture, at a lot we introduced besides BPs a concomitant course of DX, glucocorticoid, our primary interest is for patients suffering with multiple myeloma. The doses have been established based on doses administered to humans, the equivalent of 490 mg for a 70 kg patient for DX and 4 mg ZA for a 60 kg patient. For equivalence we used the Km factor described by Shannon Reagan-Shaw et al. [12].

BPs form a strong and selective bond in the mineral bone, after attaching to osteoclasts they inhibit their activity, development, migration and viability, these leading to osteoclasts apoptosis [4].

The phosphonate groups are necessary for cell-mediate antiresorptive activity and for connecting to bone mineral. Alteration of one or both phosphonate groups can significantly diminish the affinity of the BPs for bone mineral [13], and reduce biochemical potency (figure 4) [14,15].



Fig.4. Structure of bisphosphonates classified according to their biochemical mode of action by R. Graham G. Russell [16]

The nitrogen-containing BPs (N-BPs) pamidronate, alendronate, ibandronate, risedronate and zoledronate are more potent than the simple BPs (clodronate and etidronate) in inhibiting bone resorption in preclinical models [17].

The hydroxyl group represented by the R1 chain, with no antiresorptive effect, enhance chemisorption to mineral [18,19]. The R2 chain determines bisphosphonate efficacy through its antiresorptive capacity. The side chains may have different positions, so the bisphosphonates each have their own chemical, physicochemical and biological characteristics [13].

Due to the nitrogen atom within the heterocyclic ring, risedronate and zoledronate are the most potent antiresorptive BPs in several animal models [20]. Ibandronate with its more highly substituted nitrogen moiety is more potent than alendronate and pamidronate, compounds that have a basic primary nitrogen atom in an alkyl chain. The increased antiresorptive potency noticed with the different R2 groups, suggested that these N-BPs inhibit an enzyme further upstream in the mevalonate pathway, the farnesyl pyrophosphate synthase (FPPS) enzyme, and is thought to also be linked to the ability to bind to hydroxyapatite (HAP) crystals [21].

Glucocorticoids have direct adverse effects on osteoblasts, osteoclasts, and osteocytes. Through their effect on osteocytes, induce apoptosis, decrease bone strength and vascularity and could lead to osteonecrosis. The risk of osteonecrosis increases with increasing dose and duration of glucocorticoid, in particular DX (figure 5) [22].



BPs, DX, and local surgical trauma have each been identified as risk factors for osteonecrosis of the jaw. The administration of ZA+DX prior to dental surgical trauma in rats therefore lead to changes in bone and soft tissue that are similar to those noted in humans who develop BRONJ.

New bone begins to form 14 days after extraction in normal rats, showed several studies [24,25]. In present study, 14 days post-local trauma, complete epithelialization of the sites was observed only in control animals. In those animals treated with ZA+DX exposed bone was observed in less animals compared to the lot treated with ZA group were the majority of the animals presented open wounds and exposed bone (seven of eight).

Osteoclast attachment was extremely flawed in the treated group number two. This may be supported by the theory that unlike other anti-resorptive drugs, BPs physically accumulate in alveolar bone [26], and this may suggest the involvement of local mechanisms in BRONJ pathogenesis.

In ŽA group larger area of bone matrix become involved, this could be explained by the higher affinity of zoledronate to bone, which makes them more potent [27]. After dental surgical trauma, osteoclasts affected by zoledronate detach and the apoptosis occurs, leading to a delay in the healing process and related bacterial colonization, secondary bone osteonecrosis apears sooner [28]. In this conditions, bone remodeling would not start, eventually in a zoledronate-free zone were osteoclasts can attach and start separating the viable bone from the sequestration. Future studies are nedded to determine the involvement of microorganisms in the induction and production of BRONJ.

The administration of ZA+DX has clinical relevance, and delayed healing is not a usual consequence of DX use. Many patients treated for multiple myeloma receive both drugs, to minimize postoperative complications of third molar extraction DX is prescribed [29]. In other treatment indication steroid-induced osteonecrosis has been reported, but there is avascular necrosis that apears in joints [30] and it can not be compared to our model were no notable differences in vascularity were seen between control and ZA+DX group. In the ZA+DX group we demonstrated qualitative differences from controls related to integrity of overlying mucosa, number of bony sequestra, extent of the vascularity and bone cellularity. Although focal necrosis was present in two animals treated with ZA+DX, the presence of extensive osteocartilaginous regeneration through chondrocyte hyperplasia in place of bone tissue was observed. It appeared that the combination of ZA+DX was important as no ulceration was seen, in contrast with the ZA group were ulceration was sees in some samples. The character of the inflammatory infiltrate was not significantly different between the control and ZA+DX groups. This results are similar to the study conducted by S.T. Sonis et al. [31].

With this study we provide preliminary observations and histologic presentation of BRONJ and we achieved a BRONJ rat model. Empty lacunae with necrosis, poor vascularity and low number of detached osteoclasts were found in alveolar bone in ZA-treated rats. This demonstrated the effect of BPs in delaying bone turnover wich is an important factor in the development of BRONJ after local trauma.

Conclusions

The strengths of the present study is that we achieved a BRONJ model with a dose equivalent to humans but in a short time. With our model we expect to provide useful informations for further studies in the research of this severe disease. However, the present study has limits, because it was performed on a rat model, which has different anatomy and bone turnover and structure compared to humans. There is need for more clinical and chemical studies with other BPs and DX doses, timings and larger animal models or clinical situations to define BRONJ in multiple myeloma population and to provide useful evaluation and treatment options for BRONJ.

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