

Effect of Alendronic Acid on Buccal Mucosa

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Abstract

Objective: Alendronic acid, a nitrogen-containing bisphosphonate has a cytotoxic effect on oral epithelial cells. The aim of our study was to investigate the effects of alendronic acid on buccal mucosal cells and on VEGF expression.

Study design: Twenty female Wistar rats were divided into two subgroups of 10 animals, the Experimental Group and the Control Group. Alendronic acid was administered per os to animals at a dose of 0.05 mg/kg b.w /week for 13 weeks. An electron microscopy study of the mandible tissue and simultaneously, an immunohistochemical study of VEGF expression on the same tissues were performed. Mann-Whitney test has been used for statistical analysis of the study findings.

Results: Alendronic acid provoked a decrease of the epithelial cell layers in 20% of the specimens. Pyknotic nuclei were described in 40% of them, while in 70% of tissues, basement membrane was ruptured with a profound decrease of hemidesmosomes, signing the beginning of apoptosis. In 60% of tissues, an extensive edema of the dermis was also described. In accordance to these observations, VEGF expression was found increased in 60% of the tissues.

Conclusions: Alendronic acid has a potent apoptotic effect on buccal epithelial cells and affects the integrity of the dermis. The increased expression of VEGF in the experimental group supports its apoptotic action on the tissue and furthermore the healing process that follows.

Keywords: Bisphosphonates, Buccal mucosa, Electron microscopy, VEGF

Introduction

Bisphosphonates (BP), have been used effectively as a drug, due to their great affinity to osseous tissue, as a treatment in various osteopathies, such as osteoporosis, Paget's disease or osteopenia associated with myeloma or breast cancer [1,2]. Bisphosphonates (BP) bind hydroxyapatite of bone and inhibit osteoclast-mediated bone reabsorption. The main side effect of BPs is the Osteonecrosis of the Jaw (ONJ), a serious complication of bisphosphonate treatment for which the pathophysiology is unknown [3].

Alendronic acid, a second generation nitrogen containing bisphosphonate, is the potent bisphosphonate in clinical use. Alendronic acid had been proved to inhibit cell signaling, through the mevalonate pathway and by blockage of small signaling proteins, essential for normal cell function and survival [4,5]. What is more, other studies have suggested that high doses of nitrogen-containing bisphosphonates had resulted in higher levels of apoptosis and lower levels of MMP-9 in the oral epithelial cells supporting the idea of bisphosphonate treatment effect on the oral mucosa [6].

Neoangiogenesis is stimulated by VEGF (Vascular Endothelial Growth Factor), a glycoprotein with proliferative effect on the endothelial cells. VEGF acts directly on the endothelial cells through its receptors (VEGFRs) by triggering not only the proliferation of the endothelial cells, but their migration and their transformation into vessels too. VEGF expression was found to be increased in hypoxia, oncogenesis, atherosclerosis, arthritis, and tissue healing. Furthermore, alendronic acid was found to downregulate the release of VEGF in a dose dependent way [7]. Specifically, in a study with 18 post-menopausal women, a downward trend in VEGF expression was observed, at 6 and 12 months after treatment with alendronic acid [8]. Additionally, the expression of the endothelial genes CD31, VE-cadherin and VEGFR2 was down regulated by alendronic acid [9]. Last but not least, interventions with bisphosphonates were found to alter capillary regeneration [10].

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Regarding buccal mucosa, this had been considered to be a lining mucosa. The epithelium was described as thick, stratified squamous, non-keratinized and the lamina propria was found dense with short, irregular papillae along with collagen fibres. In addition to this, a submucosa layer was present with a lot of minor salivary glands, beneath which lied fibres of buccinator muscles. A mitotically active basal cell layer was also described, advancing through many differentiating intermediate layers to the superficial layers, where cells were shed from the surface. Buccal mucosa provides a selectively permeable barrier between the contents of the gastrointestinal tract and the tissues of the body [11]. Several studies had previously examined the effects of bisphosphonates on cells of the oral cavity. The therapy with systemic bisphosphonates to treat osteoporosis caused brown dermal nodules on a few patients' malar cheeks bilaterally [12]. Furthermore, a toxic effect on the buccal mucosa was reported during clinical examination [13], when oral ulcers were noticed [14].

The aim of our report is to enlighten the histological effect of systematic alendronic acid administration in oral mucosa. In order to enhance our results, a further immunohistochemical investigation of VEGF expression in buccal mucosa was performed in an in vivo experimental animal model.

Materials and Methods

In order to perform the experiment, we handled twenty female Wistar rats, 12 months old, weighing approximately 500 grams each. The rats were housed in stainless steel cages, with one rat per cage, 12 hour light-dark cycle, and controlled humidity and temperature. The animals were randomly divided into two groups: Group A, the experimental group that consisted of 10 animals, and Group B, the control group that also includes 10 animals. Alendronate was administered per os to animals of Group A at a dose of 0.05 mg/kg body weight / week dissolved in 3cc normal saline for 13 weeks, as previously described. The drug was administered thirty minutes before feeding. The usual human dosage was used in order to calculate the exact amount of drug given [15,16]. The duration of the study was limited to 13 weeks, due to the fast accumulation of alendronic acid in bone tissues. After euthanasia, the mandible of the animals was removed and specimens of the buccal mucosa were taken. Finally, tissues were processed for examination with the optical and the electron microscope.

Transmission Electron Microscopy

Mandibular tissue samples were sectioned into pieces with size below 1 cm³. Afterwards, they were placed into glutaraldehyde 3% for 2 hours, then into osmium tetroxide (OsO₄) 1% for 1 hour. The staining process was performed with uranyl acetate 1% for 16 hours. Then, samples were dehydrated with increased ethanol concentrations. The next step was to embed sample tissues into Epon resin and to cut ultra-thin sections (600–900 Å). Finally, sections were stained with Reynolds's stain. Samples were observed with a Transmission Electron Microscopy (type JEOL 1011).

Immunohistochemistry

Mandibular tissue sections from both groups were embedded in paraffin and were cut into thin sections of 3-4 µm. The

unstained specimens were processed using the kit Polymer Detection System (Novolink, Novocastra) with the appropriate procedures. First, deparaffinization was performed in xylene. Afterwards, specimens were immersed in absolute alcohol, in degressive densities 100%, 96% and 70% v/v consecutively. Finally, they were rinsed with distilled water. Antigen retrieval was performed by incubation at 650 °C. Following this, specimens were first rinsed with PBS buffer, then incubated in H₂O₂ for 5 min to quench endogenous peroxidase activity and finally, rinsed again with PBS buffer. Thereafter, specimens were covered with a solution of the primary tonic monoclonal antibody against Human Vascular Endothelial Growth at a concentration of 1/50 (VEGF, clone VG1, Dakocytomation. At last, they were washed using WAS solution.

For the detection of immunohistochemical staining, specimens were immersed in Post-Primary solution. After being washed, they were immersed in polymere solution and then in chromogen-diaminobenzidine (DAB) solution. Afterwards, they were stained with Haematoxylin- Eosin. Last but not least, they were rinsed in tap water and dehydrated with escalating densities of ethanol solution and xylene (70, 96 and 100% v/v consecutively). Finally, they were covered with tape, placed in glass plates and immersed in Canada balsam. Previously reported immunohistochemical staining procedure was repeated twice. Specimens were examined with an optical microscope (Zeiss) and photographs were taken with a camera (Contax), attached to the microscope. Intensity of staining was evaluated with a qualitative method (Mann-Whitney test) as negative (-), weak (+), moderate (++) and strong (+++) by two independent reviewers.

Results

Macroscopic examination

By the macroscopic examination of the mandible, ulcers did not occur in any rat either in the control group or the experimental group. In addition to that, two rats presented lesions with a slight whitish color, indicating an increased keratinization, according to five observers of our laboratory staff.

Transmission electron microscopy studies

In the control group the mucosa was normal in 100% of sections (Table 1). Epithelium was noticed to be the typical not keratinized squamous, with a continuous basement membrane that connects with epithelium cells with many hemidesmosomes. The cells of the stratum spinosum had bundles of tonofilaments that ended in the abundant desmosomes of the cells. The lamina propria was dense with bundles of collagen fibers and fibroblasts (Figure 1). Regarding the experimental group, a variety of morphological changes were noticed among samples. Epithelium was normal not keratinized squamous, in 8 out of 10 animals (80%). However, in 2 animals (20%) (Table 1) a keratinized epithelium was observed (Figure 2). In 2 out of 10 animals (20%) of the experimental group, a decrease of the cell layers was observed (Figure 3) with no cytoplasmic procedures of the basal stratum (Table 1). No mitotic cells were noticed. Basement membrane in seven animals (70%) (Table 1) was ruptured and the number of the hemidesmosomes was found decreased (Figure 4). In the papillary layer of the dermis and specifically in the reticular layer, in 6 out of 10 animals (60%) (Table 1), only a few bundles of collagen fibers were noticed, with

Table 1: Pathological inflammatory alterations of buccal mucosa cells after ALN administration.

Pyknotic (apoptotic) nucleus of epithelial cell	Mast cell	Active fibroblast	Bundles of collagen fibers in different directions and edema	Rupture of the basal lamina and decrease in the number of the hemidesmosomes	Decrease of epithelial cell layers.	Keratinization of epithelium		GROUP
0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	YES	Control group
10(100%)	10(100%)	10(100%)	10(100%)	10(100%)	10(100%)	10(100%)	NO	
4(40%)	2(20%)	2(20%)	6(60%)	7(70%)	2(20%)	2(20%)	YES	Experimental group
6(60%)	8(80%)	8(80%)	4(40%)	3(30%)	8(80%)	8(80%)	NO	
20	20	20	20	20	20	20		SUM

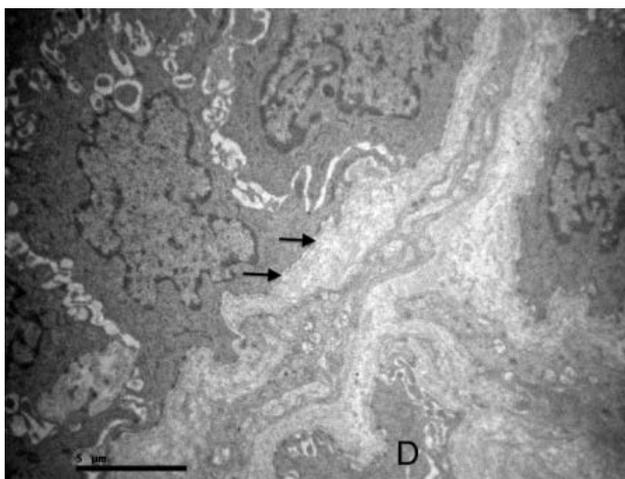


Figure 1: Control group: Basal lamina and hemidesmosomes (arrows). Dermis (D).

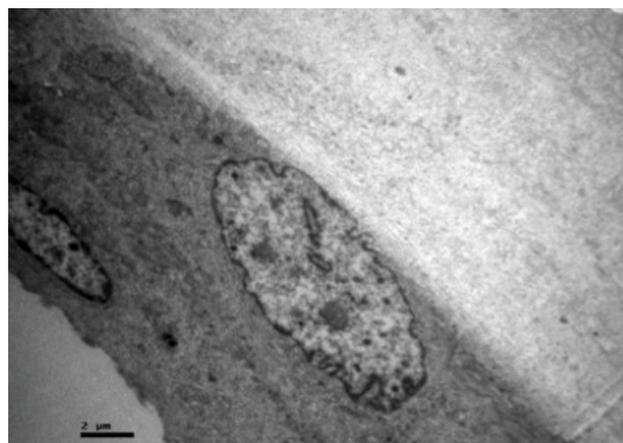


Figure 3: Experimental group: The decrease of the epithelial cell layers, with no cytoplasmic procedures.

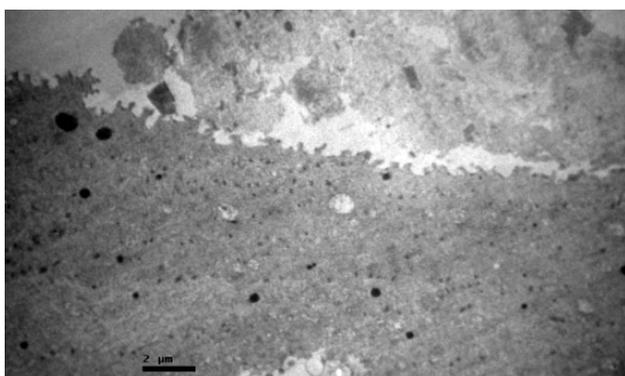


Figure 2: Experimental group: Keratinization of the epithelium.

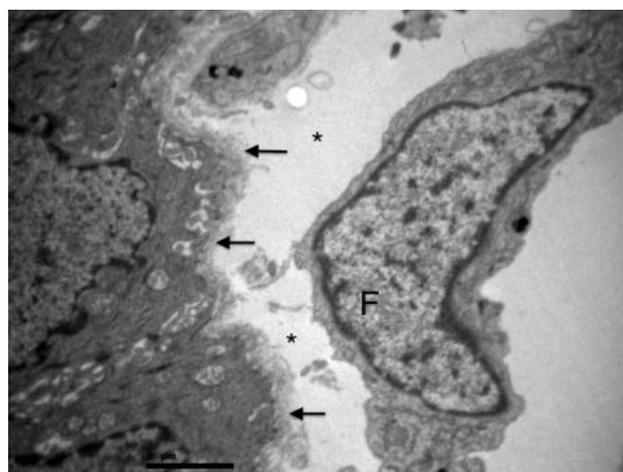


Figure 4: Experimental group: Rupture (first, second arrow) of the basal lamina and a decrease in the number of the hemidesmosomes (third arrow). Dermis without collagen fibers (*). Fibroblast (F).

abundant extra matrix substances, thus indicating the presence of extensive edema (Figure 5). Active fibroblasts were found in only two animals (20%) from the experimental group (Figure 6). Mast cells were also described in two animals (20%) from the experimental group (Table 1) (Figure 5). Last but not least, apoptotic (pyknotic) nucleus was noticed in 4 out of 10 animals (40%), from both epithelial cells and fibroblasts (Figure 5,7).

Immunohistochemical staining for VEGF

In 6 out of 10 animals of the experimental group (60%) (Table 2), a weak(+) positive expression of VEGF in dermis was noticed.

Experimental group	Control Group	VEGF expression
4(40%)	10(100%)	negative (-)
6(60%)	0(0%)	weak (+)
0(0%)	0(0%)	moderate (++)
0(0%)	0(0%)	strong (+++)
10(100%)	10(100%)	SUM

Table 2: Expression of VEGF in buccal mucosal tissue after administration of ALN.

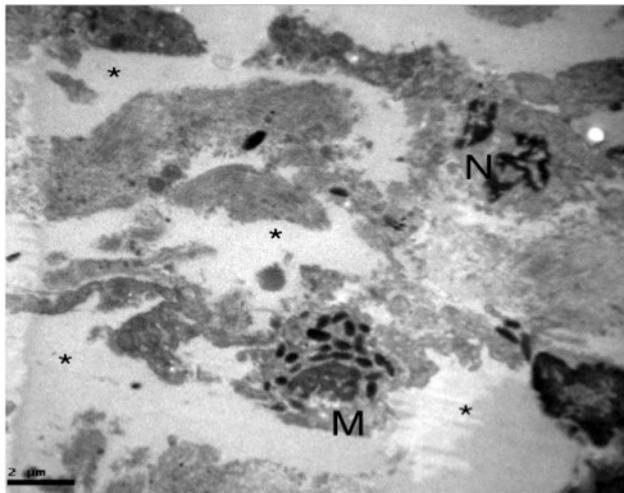


Figure 5: Experimental group: A mast cell (M), a cell with a pyknotic nuclei (N) and extensive edema (*) in dermis.

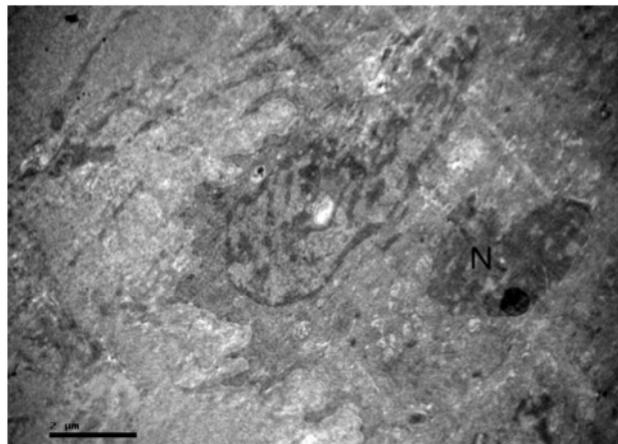


Figure 7: Experimental group: The apoptotic nucleus (N) of an epithelial cell.

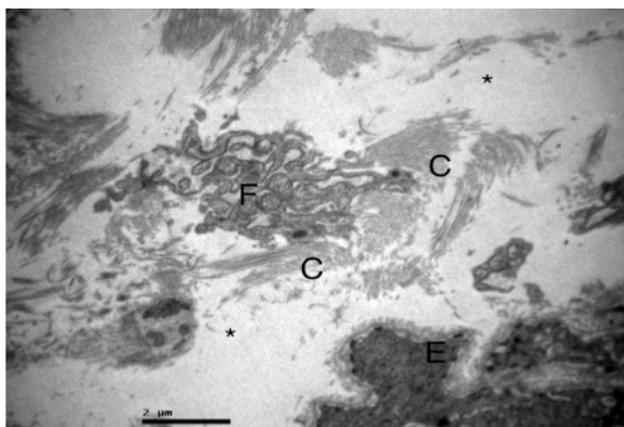


Figure 6: Experimental group: Dermis with few bundles of collagen (C) fibers and abundant extramatrix substances. Edema (*). Epithelium (E). An active fibroblast (F).

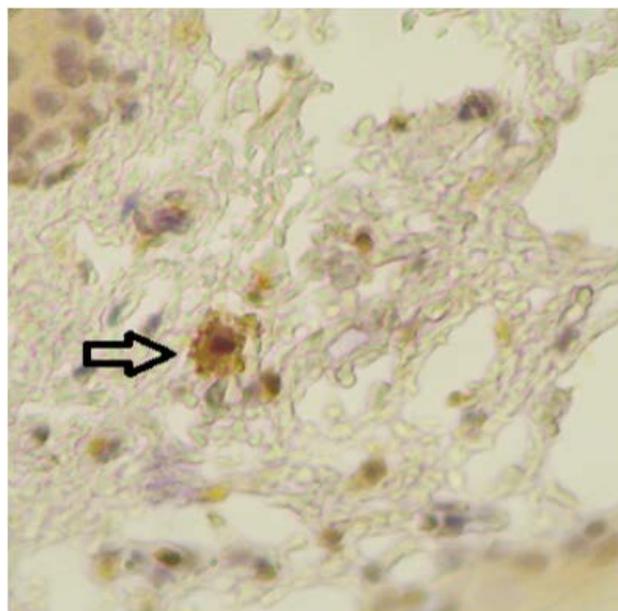


Figure 8: Experimental group, Immunohistochemical examination: A lightly positive expression of VEGF in dermis in the experimental group. VEGF is expressed both in the cell nucleus and cytoplasm.

In 4 out of 10 sections (40%) no VEGF expression was found (Figure 8). On the other hand, in the control group, none of the sections (100%) were stained positive for VEGF.

Discussion

In this study, we have investigated the morphological changes on buccal mucosa after alendronic acid administration. Furthermore, we have studied the levels of VEGF, as a marker of neovascularization and healing process of the tissue. In a previous study, we had suggested that treatment with bisphosphonates may cause ultrastructural alterations of the inferior alveolar nerve, using the same method [17].

Toxic effects after the administration of bisphosphonates had been reported in previous studies. A possible relationship between the occurrence of oral ulcers and the use of oral bisphosphonates was documented [14,6]. A case report with a patient with ulcerating lesion of the upper alveolus had suggested that oral bisphosphonates may cause avascular necrosis [18]. Regarding

the macroscopic examination of the animals in our experiment, no ulcers were observed. However, in two specimens, lesions with a slight whitish color were noticed, indicating an increased keratinization. What is more, in microscopic examination, rupture of basal lamina was detected in 70% of the tissues. During the electron microscopy study, an approximately 20 percent from the specimen had a keratinized epithelium (Table 1). Previous results agree with bibliography, which supported that bisphosphonates decrease cell viability, migration ability, and increase apoptosis rate at the oral mucosa [19].

Moreover, alendronic acid was also associated with an anti-proliferative effect on the oral epithelium. Specifically, human gingival fibroblasts lines and human keratinocyte cells lines were

noticed to “ball-up”, then to fragment and finally to show dead floating cells, indicating apoptosis, in a dose dependent manner. According to another research, high doses of bisphosphonates resulted in higher levels of apoptosis and lower levels of MMP-9 in oral epithelial cells [6]. Our results were in agreement with these findings. In our study, nucleus’s fractions and active fibroblasts were observed in the epithelium from 40% of tissues (Table 1). In addition to this, in another 60% (Table 1) tissue edema was observed and bundles of collagen fibres were found broken after the administration of bisphosphonates. Last but not least, in a 20 percent, mast cells were found in the connective tissue (Table 1), indicating a possible inflammatory process. However, no lymphocytes or polymorphism cells were observed. These findings were in agreement with previous reports, claiming that there is a positive correlation between the number of mast cells and the degree of inflammation [20]. A downward trend in the number of hemidesmosomes was also described after alendronic acid administration. This could indicate the formation of ulcerous lesions in buccal mucosa. A potential negative effect of alendronic acid administration in tissue structure and cell function was obviously described.

In general, VEGF expression has been proved to increase during tissue healing process. A decreased expression of this factor could inhibit the neoangiogenesis and the reconstruction of buccal mucosa [21]. In our study, we performed an immunohistochemical study of VEGF expression in tissues after alendronate administration. VEGF expression was found positive in 60% of tissues (Table 2). Our results were in complete disagreement with previous research. Specifically, one study suggested that administration of nitrogen-containing bisphosphonates should be withheld in patients with ischemic events, such as acute limb ischemia, due to its potential actions against neoangiogenesis [22]. However in this report, only administration of zoledronate was studied. VEGF expression was found upregulated after alendronic acid administration, proposing the start of a healing process after alendronate apoptotic actions on the tissues.

Consequently, we demonstrated that alendronic acid induces an inflammatory process on buccal mucosa, both in cells and tissues, possibly as a response at its apoptotic effect. Increased VEGF expression was also observed, indicating that alendronic acid provokes tissue healing. However, the duration of our experiment was relatively short and latter pathological findings in cells and tissues after alendronate administration were possibly not observed. Further investigation is needed to investigate the extent of pathological inflammatory and apoptotic changes of alendronic acid in the tissues.

Ethics

This research complies with the guidelines for animal studies. The study protocol has been approved by the Bioethics Committee of the Medical School of the Aristotle University of Thessaloniki and thus meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996 [JAMA 1997;277:925-926]. Further, this animal experiment conforms to our institutional standards.

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Disclosure statement

The authors have no conflicts of interest to declare.

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