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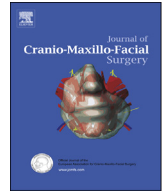

# The influence of geranylgeraniol on human oral keratinocytes after bisphosphonate treatment: An in vitro study

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ABSTRACT

This in vitro study analyzed the influence of geranylgeraniol (GGOH) on human oral keratinocytes (HOK) after exposure to bisphosphonates.

HOK were incubated with four different bisphosphonates (clodronate, ibandronate, pamidronate, zoledronate) in two experimental set-ups: with and without GGOH. MTT and PrestoBlue assays were used to analyze HOK cell viability. HOK migration ability was examined with Boyden and Scratch assays, and Tunel and ToxiLight assays were used to detect the HOK apoptosis rate.

No significant differences between the experimental set-ups, with and without GGOH, could be found for clodronate ($p$ each $>0.3$). For the nitrogen-containing bisphosphonates, negative effects could be shown in the experimental set-ups without GGOH in all assays. In the GGOH experimental set-ups, the levels of HOK cell viability were significantly increased (MTT: $p$ each $\leq 0.001$; PrestoBlue: $p$ each $\leq 0.012$). The HOK migration ability was also greater (Boyden: $p$ each $<0.001$; Scratch: $p$ each $\leq 0.015$). Regarding the apoptosis rate, reduced numbers of apoptotic HOK in the Tunel assay ($p$ each $<0.001$) and decreased adenylate kinase release in the ToxiLight assay ($p$ each $\leq 0.002$) were observed.

GGOH reversed the negative effects of bisphosphonates on HOK. These findings provide evidence that GGOH could be a promising treatment option for BP-ONJ.

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## 1. Introduction

In modern medicine, bisphosphonates play a key role in the therapy of osseous malignancies, such as multiple myeloma, and osteoblastic and osteolytic metastases, as well as metabolic bone diseases (Neville-Webbe and Coleman, 2010). In addition to hypocalcemic episodes, acute phase reactions, gastrointestinal and renal side effects, bisphosphonate-associated osteonecrosis of the jaws (BP-ONJ) was reported by Marx for the first time in 2003 (Marx, 2003; Diel et al., 2007).

Even a decade later, the exact pathophysiology of BP-ONJ has not been clarified in detail, and different theories are being discussed (Allan and Burr, 2009). The most commonly cited theory describes the influence of bisphosphonates on osteoblasts and osteoclasts resulting in a consecutively modified and reduced bone-remodeling (Mashiba et al., 2005; Walter et al., 2010; Koch et al., 2011a; Açil et al., 2012). In addition, a strong antiangiogenic potency of bisphosphonates has been demonstrated which might be an important factor in BP-ONJ pathophysiology (Walter et al., 2010, 2011; Ziebart et al., 2011a; Pabst et al., 2014). Concerning the relevance of angiogenesis and vascularization in BP-ONJ development, Wehrhan et al. (2011a) analyzed and differentiated angiogenesis and vascularization in human BP-ONJ-adjacent mucoperiosteal tissue specimens after zoledronate therapy. This study demonstrated, that the remodeling of vessels, as well as the formation of neovessels, is significantly delayed and impaired in BP-ONJ tissues, while vascularization and mature capillary density remains unaffected (Wehrhan et al., 2011a). Further, as previously summarized and reported by Wehrhan et al., it has been demonstrated that N-BP treatment is able to increase vascularization of avascular bone in patients with bone necrosis and to speed up fracture healing in extracranial skeletal bone (Agarwala et al., 2002; Matos et al., 2007; Amanat et al., 2007; Wehrhan et al., 2011b).

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Hence, the pathophysiological significance of reduced vascularization for BP-ONJ development and the characterization of BP-ONJ as an 'avascular necrosis' remains the subject of discussion.

Further, the strong influence of bisphosphonates on the immune system has been reported (Hagelauer et al., 2014a). More recently, it has also been shown that patients with periodontal diseases seem to be particularly at increased risk of BP-ONJ development. In this context, periodontal disease therapies for patients who are about to receive bisphosphonates should be strictly adhered to (Walter et al., 2014a).

Some research efforts have increasingly focused on the soft oral tissues, especially on oral keratinocytes, in relation to the pathophysiology of BP-ONJ; this is because sufficient wound healing often fails, especially in regions with a very thin mucosal layer, such as the mylohyoid line (Reid et al., 2007). In this context, oral keratinocytes are the most important cell line of the oral mucosa and the keratinized gingiva. These cells create a covering epithelial keratinocyte layer over the structural framework (stroma); which is created by gingival fibroblasts and completed by endothelial cells, which are the cell line responsible for vascularization. It is well known that oral keratinocytes are negatively influenced by nitrogen-containing bisphosphonates in particular (Pabst et al., 2012). This might explain the clinical picture of a non-healing wound in combination with exposed, necrotic and bacterially contaminated underlying bone, leading to the development of BP-ONJ (Allan and Burr, 2009; Walter et al., 2010, 2011).

Concerning BP-ONJ therapy, preventive strategies and conservative management of BP-ONJ are possible, with surgical treatment including debridement and resection of the affected regions with a plastic and saliva-proof wound closure (Van der Wyngaert et al., 2009; Williamson, 2010). Although preventive strategies are increasingly implemented for BP-ONJ, the frequency of new cases is still increasing. Unfortunately, most current prevention strategies used have not reduced the frequency of new cases (Diel et al., 2007; Walter et al., 2007, 2014b). It is still unclear if the lack of reduction in new cases is due to increasing numbers of patients receiving bisphosphonates or due to the preventive strategies being ineffective. Significant progress has been made in the diagnosis and intraoperative visualization of BP-ONJ with the use of computed tomography and fluorescence imaging (Assaf et al., 2014; Hamada et al., 2014). Supportive therapeutic options such as hyperbaric oxygen and soft laser therapy have not lived up to expectations (Freiberger et al., 2007, Freiberger, 2009; Vescovi et al., 2010). The positive benefit of teriparatide therapy in order to prevent and treat BP-ONJ has only been investigated in a rat model (Ersan et al., 2014). Dexrazoxane hydrochloride has been reported to have cytoprotective effects on zoledronate-treated osteoblasts and fibroblasts as well as on drill-hole defects in zoledronate-treated rabbits (Draenert et al., 2012). These results do not allow us to draw conclusions in relation to the treatment of patients. Consequently, there is a pressing need for new therapeutic strategies.

Previously, a beneficial effect of isoprenoid geranylgeraniol on bisphosphonate-treated monocytes has been described (Marcuzzi et al., 2010). In addition, it has been shown that geranylgeraniol can reverse the bisphosphonate-induced inhibition of cell viability and the migration ability of fibroblasts, osteogenic and endothelial cells (Ziebart et al., 2011b). These effects are based upon the pharmacological mode of action of nitrogen-containing bisphosphonates, inhibiting the enzyme farnesyl pyrophosphate synthase and thereby the synthesis of the geranylgeraniol as a key substrate in the mevalonate pathway. For a detailed overview about the exact mechanisms, see the following references (Casey, 1992; Shipman et al., 1998; Virtanen et al., 2002; Reszka and Rodan, 2003; Walker and Olson, 2005; Ziebart et al., 2011b; Hagelauer et al., 2014b).

Since there is no effective preventive therapy for BP-ONJ and due to the relevance of oral keratinocytes in BP-ONJ development, the aim of this study is to analyze the effects of geranylgeraniol on cell viability, migration ability and the apoptosis rate of human oral keratinocytes after bisphosphonate exposure in vitro.

## 2. Materials and methods

### 2.1. Cell culturing

HOK (human oral keratinocytes; ScienCell, Carlsbad, CA, USA) were cultured in keratocyte growth medium (Provitro, Berlin, Germany) supplemented with 500 ng/500 ml medium bFGF (basic fibroblast growth factor), 10 ng/ml EGF (epidermal growth factor; recombinant human), 5 μg/ml insulin (recombinant human), 0.004 ml/ml BPE (bovine pituitary extract) and 1 μg/ml hydrocortisone. HOK were incubated in a cell culture incubator with 5% $CO_2$ and 95% air at 37 °C. HOK were passaged at regular intervals depending on their growth characteristics using 25% trypsin (Seromed Biochrom, Berlin, Germany).

### 2.2. Bisphosphonates and geranylgeraniol

One non-nitrogen bisphosphonate (NN-BP) (clodronate: Bonefos, Bayer AG, Leverkusen, Germany) and three nitrogen-containing bisphosphonates (N-BPs) (ibandronate: Bondronat, Roche, Mannheim, Germany; pamidronate: Pamifos, Medac, Wedel, Germany; and zoledronate: Zometa, Novartis, Nürnberg, Germany) were used. The bisphosphonate doses used represent the concentrations during patient treatment (Otto et al., 2010). The concentrations of BP and GGOH (geranylgeraniol; Sigma, München, Germany) have been established previously (BP: 0, 5, 50 μM; GGOH: 10 μM) (Freiberger et al., 2007; Walter et al., 2010, 2011; Ziebart et al., 2011a, 2011b; Pabst et al., 2014).

### 2.3. HOK cell viability

#### 2.3.1. MTT assay

An MTT colorimetric assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide; Sigma, München, Germany) was used to analyze HOK cell viability. HOK cells convert tetrazolium bromide into formazan, which can be measured photometrically after cell lysis with a Synergy HT Multi-Mode Microplate Reader (corresponding setting: absorbance; BioTek Instruments, Winooski, VT, USA).

HOK were seeded into 6-well plates (250,000 cells/well). After 24 h, HOK were incubated with the different bisphosphonates (clodronate, ibandronate, pamidronate, zoledronate) at different concentrations (0, 5, 50 μM) and GGOH (10 μM) for 72 h. The control groups received no GGOH.

#### 2.3.2. PrestoBlue assay

PrestoBlue assay (Invitrogen, Darmstadt, Germany) was used to analyze HOK cell viability and proliferation ability. After applying the PrestoBlue reagent, the color change induced by the reduction of resazurin to resorufin can be measured at a wavelength of 560/20 and 620/40 nm with a Synergy HT Multi-Mode Microplate Reader (corresponding setting: fluorescence; BioTek Instruments, Winooski, VT, USA).

6-well plates were prepared similarly to the MTT assay (250,000 cells/well). After 24 h, HOK were incubated with the mentioned bisphosphonates in different concentrations (0, 5, 50 μM) and GGOH (10 μM). The control group received no GGOH. PrestoBlue assay was measured after 24, 48 and 72 h.

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