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內文：

Abstract

- ✓ **Bisphosphonate-associated osteonecrosis of the jaws (BP-ONJ)** is one of the main side effects
- ✓ The exact mechanism of action and etiopathology is still **unknown.**
- ✓ In addition to inhibition of bone remodelling, an **anti-angiogenetic effect** has become the focus of research
- ✓ The aim of these study was to investigate the effect of **different bisphosphonates** on human **umbilicord vein endothelial cells (HUVEC)** and **endothelial progenitor cells (EPC)**, which play an important role in angiogenesis.
- ✓ Using varying concentrations, the impact of one **non-nitrogen** containing bisphosphonate (clodronate) and three **nitrogencontaining** bisphosphonates (ibandronate, pamidronate and zoledronate) on HUVEC and EPC was analysed.
- ✓ The biologic behaviour of HUVEC after incubation with different bisphosphonates was measured in a Boyden **migration assay** as well as in a 3D **angiogenesis assay**. The number of **apoptotic cells** was measured by Tunnel assay.
- ✓ To underline the importance of neoangiogenesis in the context of BP-ONJ, we measured the **EPC number** after incubation with different bisphosphonates in vitro
- ✓ HUVEC and EPC were significantly influenced by bisphosphonates at different concentrations compared with the non-treated control groups.
- ✓ The **nitrogen-containing** bisphosphonates pamidronate and zoledronate had the greatest impact on the cells, whereas **clodronate** followed by ibandronate was less distinct on cell function.
- ✓ These results underline the hypothesis that inhibited **angiogenesis induced by bisphosphonates** might be of relevance in the development and maintenance of BP-ONJ.

Introduction

- ✓ **The benefit of bisphosphonates** in the treatment of malignant bone neoplasias like multiple myeloma, bone metastases or metabolic bone diseases like Paget's disease and severe osteoporosis is without controversy.
- ✓ Since 2003, a new specific side effect, bisphosphonate-associated osteonecrosis of the jaws (BP-ONJ), has increasingly become the focus of clinical and preclinical investigations.
- ✓ BP-ONJ is defined as exposed necrotic bone in the maxillofacial region for a

period of **at least 8 weeks** in connection with current or previous bisphosphonate therapy and a lack of head and neck radiation in the patient's history.

- ✓ **Nitrogencontaining bisphosphonates** such as pamidronate and zoledronate are more often associated with BP-ONJ compared to the less potent ibandronate and the nonnitrogen- containing clodronate.
- ✓ The reduced bone remodelling in BPONJ patients is attributed to bisphosphonate-induced **inhibition of osteogenic cells and osteoclasts** in a dose-dependent manner. Furthermore, bisphosphonates have been proven to reduce viability of oral keratinocytes, which corresponds with **impaired mucosal wound healing**
- ✓ Bone tissue is particularly vascularised, and **endothelial cells** have been proven to play an essential role during bone remodeling
- ✓ the term **angiogenesis** refers to sprouting from preexisting vessels of rather mature endothelial cells. Of great scientific interest is the fact that bone-marrow-derived, circulating **endothelial progenitor cells (EPC)** bear the potential of creation of primordial vessels, called **vasculogenesis**
- ✓ EPC have the potential to differentiate into **mature endothelial cells.** Furthermore, EPC show strong **paracrine effects** by production of several cytokines and growth factors which increase neovascularisation
- ✓ However, **histopathological** analysis of BP-ONJ bone specimens revealed markedly **reduced density of vessels or even avascular necrosis.**
- ✓ The focus of the present study was to monitor the influence of four differently potent bisphosphonates on EPC **viability**, **migration and apoptosis rate**. Furthermore, mature endothelial cells (human umbilical vein endothelial cells, HUVEC) were investigated accordingly to monitor possible influence of the **maturation stage** of the endothelial lineage.

Materials and methods

-Cell culture-

- ✓ HUVEC were cultured in an **endothelial basal medium (EBM)** supplemented with 1 µg/mL hydrocortisone, 12 µg/mL bovine brain extract, 50 µg/mL gentamicin, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor and 10% foetal calf serum (FCS) until the third passage

-EPC culture assay-

- ✓ Mononuclear cells (MNCs) were isolated by density gradient centrifugation with Biocoll (Biochrom KG, Berlin, Germany) from peripheral blood of healthy human volunteers
- ✓ Immediately following isolation, total MNCs (8×106 cells/mL medium) were plated on 25 cm2 culture flasks coated with human fibronectin (Sigma, Steinheim, Germany) and maintained in EBM supplemented with EGM SingleQuots, VEGF (100 ng/mL), and 20% FCS.

-Apoptosis assay-

- ✓ HUVECs (5×104) in EBM-2 medium were seeded in sixwell plates; after 24 h, HUVECs were incubated with bisphosphonates (clodronate, ibandronate, pamidronate and zoledronate) at **increasing bisphosphonate concentrations** (0, 5, 50, 100, 200 and 500 µmol) for 24 h

-Migration assay-

- ✓ To examine the effect of bisphosphonates on HUVEC migration, we used a 24-well Boyden chamber assay system (ThinCert™)
- ✓ Cells were harvested, washed twice in PBS and resuspended in HUVEC medium for adjustment to a final concentration of 106mL−1.

- ✓ HUVECs were stimulated to **migrate from the upper to lower chambers** by the addition of 10 ng/mL VEGF to the lower chambers. After 12 h, cells were stained with fluorescent dye calcein- AM.

-In vitro angiogenesis assay-

- ✓ **Different concentrations of bisphosphonates** were added to the test medium. The sprouting colonies were photo-documented over 48 h. For measurement of the number, length and area of the sprouts, we utilised a newly designed automatic analyzing programme (CellAnalyser).

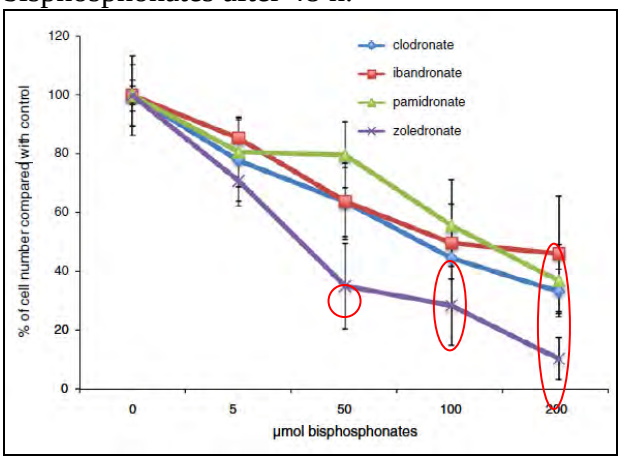
-Statistical analysis-

- ✓ Continuous variables are expressed as mean ± SEM. Comparisons between groups were analysed by t test (twosided) or ANOVA (post hoc test: Tukey) for experiments with more than two subgroups (SPSS software). **Values of $p < 0.05$ were considered as statistically significant**.

Results

-EPC count-

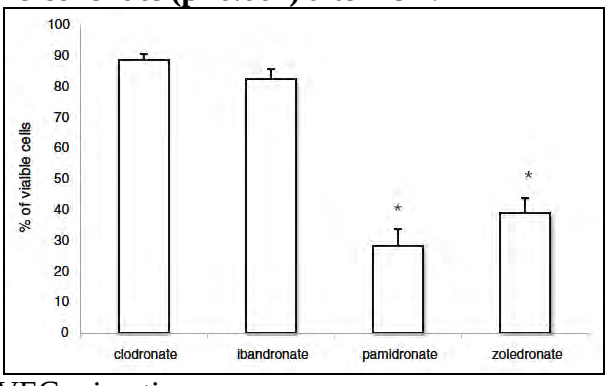
- ✓ EPC count was reduced in a dose-dependent manner for all investigated bisphosphonates after 48 h.



- ✓
- ✓ **Cell count : Zoledronate had the greatest impact.**

-HUVEC viability-

- ✓ HUVEC viability was significantly reduced for **pamidronate ($p<0.001$) and zoledronate ($p<0.001$)** after 48 h.



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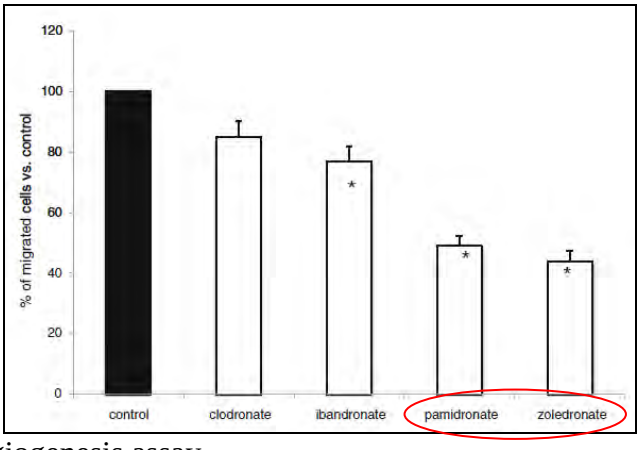
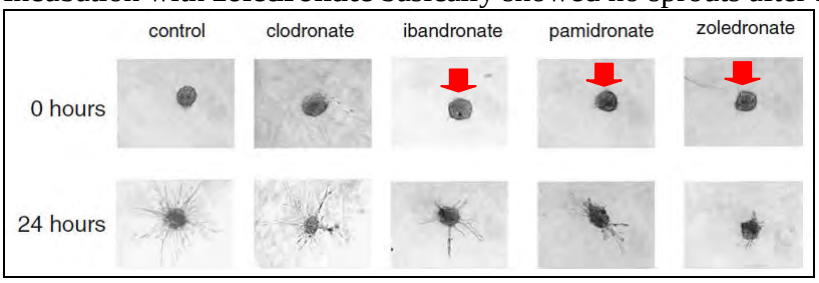
-HUVEC migration-

- ✓ The effect of pamidronate and zoledronate was significantly stronger compared to the other bisphosphonates

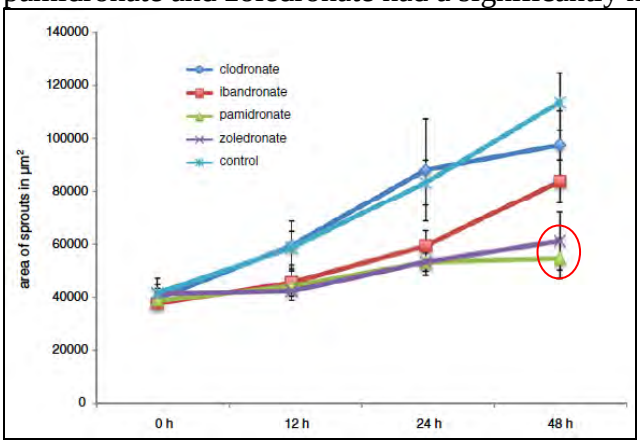
✓ 

-Angiogenesis assay-

✓ **ibandronate and pamidronate showed markedly reduced** sprouts after 48 h. Incubation with **zoledronate** basically showed no sprouts after 24 h.

✓ 

✓ pamidronate and zoledronate had a significantly negative impact at 48 h

✓ 

-Discussion-

✓ The development of blood vessels is essential for **healing and regeneration processes**, especially in the avascular necrosis of BP-ONJ

✓ EPC are important for vasculogenesis, a mechanism of new vessel formation

✓ **Nitrogen-containing bisphosphonates have an influence on the migration ability of HUVEC**

✓ HUVEC treated with **pamidronate** and **zoledronate** had a higher rate of apoptosis compared to the groups treated with clodronate or ibandronate.

✓ In the 3D angiogenesis model, **pamidronate** and **zoledronate** had significantly reduced numbers and areas of sprouts.

✓ EPC cultures treated with bisphosphonates, especially **zoledronate**, had significantly reduced cell numbers.

✓ Due to the accumulation of bisphosphonates in bone and its **long half-life,** it may interact with the release of EPCs from bone marrow niches and therefore **support the development and maintenance of BP-ONJ.**

- ✓ The biological ability, cell function and viability of HUVEC and EPC is influenced by the **nitrogen-containing** bisphosphonates ibandronate, pamidronate and zoledronate. Interestingly, clodronate has only a minor effect.
- ✓ In vitro studies on mature endothelial cells have shown impairment of cell proliferation , promotion of programmed cell death and inhibition of capillary tube formation by **bisphosphonates**
- ✓ **Non-nitrogen-containing bisphosphonates** such as clodronate are **built into** non-hydrolysable analogues of adenosine triphosphate (ATP) that inhibit many different ATP-dependent intracellular enzymes. Therefore, **very high concentrations** are needed to completely inhibit special pathways.
- ✓ In contrast, the nitrogencontaining bisphosphonates, e.g. pamidronate and zoledronate, **inhibit a key enzyme of the mevalonate pathway**: the farnesyl pyrophosphate synthase. This pathway is **important for the production of small Gproteins**, which are important for intracellular structure and mechanisms such as intracellular transport
- ✓ This may contribute to the greater impact of nitrogen-containing bisphosphonates
- ✓ With the natural limitations of in vitro studies, these data **support the theory** of the anti-angiogenetic component (angiogenesis and vasculogenesis) in the development and maintenance of BP-ONJ.
- ✓ Therefore, these findings may also **explain** the higher occurrence of BP-ONJ in patients receiving nitrogen-containing bisphosphonates as compared to patients receiving non-nitrogen-containing bisphosphonates like clodronate.

題號	**題目**
1	對於osteonecrosis of the jaw，何項錯誤？ **(A)** IV較口服容易得到ONJ **(B)** 服用corticosteroid亦是危險因子 **(C)** 若牙科治療有牽涉到齒槽骨則會提高風險，抽菸喝酒則較無相關性 **(D)** 對於ONJ的發生理論有許多，抑制血管再生乃為其中一項
答案(C)	**出處：**Bisphosphonates and Osteonecrosis of the Jaw, 2011 Nov 8.
題號	**題目**
2	**關於**雙磷酸鹽類何項錯誤？ **(A)** 雙磷酸鹽類可抑制osteoclast的作用 **(B)** 雙磷酸鹽類可以治療一些cancer，如multiple myeloma，bone metastasis **(C)** 雙磷酸鹽類對胃刺激性大，需伴隨飯後食用 **(D)** 不含氮基的雙磷酸鹽類相對需較大濃度才有效用
答案(C)	**出處：**Bisphosphonates and Osteonecrosis of the Jaw, 2011 Nov 8.