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Invited paper

Bactericidal efficacy of cold plasma in processed bone. A new approach for adjuvant therapy of medication-related osteonecrosis of the jaw?

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ABSTRACT

Background: Medication-related osteonecrosis of the jaw is a difficult to treat side effect of antiresorptive therapy. Despite intensive efforts in the treatment of this disease, therapy continues to be prolonged, burdensome and sometimes insufficient. Since bacterial contamination of the necrotic bone is a major factor in these problems, we have examined the antibacterial effect of physical cold atmospheric pressure plasma on contaminated bone.
Methods: A total of 72 cortico-cancellous porcine bone cylinders were processed and incubated in *Streptococcus mitis* broth and afterwards treated with cold atmospheric plasma, chlorhexidine or sodium chloride solution (control). After grinding up the specimens, the bacteria were transferred to aqueous solution and a colony-forming unit count was performed.
Results: Physical cold atmospheric pressure plasma showed the best results in terms of reduction in colony-forming unit count in porcine bone: overall median in colony-forming unit/ml was significantly below that for chlorhexidine ($p=0.004$) and control ($p=0.008$).
Conclusion: These results suggest that cold atmospheric pressure plasma has profound effectiveness on the bacterially contaminated bone in vitro. The use on cancellous bone is possibly a promising technique and could be a useful tool in the therapeutic range for the treatment of medication-related osteonecrosis of the jaw.

1. Introduction

Antiresorptive drugs are used widely in treating benign bone disease (e.g., postmenopausal osteoporosis, osteogenesis imperfecta, Paget's disease) as well as malignancies, such as breast, pancreatic, prostate, or lung cancer for the prevention and treatment of bone metastases or tumor-associated hypercalcemia. A feared complication is medication-related osteonecrosis of the jaw (MRONJ). Although the first cases of necrosis were registered under the use of bisphosphonates years ago [1] the pathophysiological processes that lead to the osteonecrosis of the jaw are still not fully understood. The effect of drugs on bone metabolism seems to be an important factor. Their influence on the physiological processes in the bone is manifold. Different working groups have shown drugs influence on osteoclast and osteoblast function, and also on mesenchymal stem cells [2–4]. There is also a known negative effect of bisphosphonates on blood vessels: several experimental studies have demonstrated negative influences on vascular cells and endothelial progenitor cells from nitrogen-containing bisphosphonates and on the production of local angiogenesis factors [5–7]. In addition to the bisphosphonates, other antiresorptive drugs with compromising influence on the jaw have also appeared. The human monoclonal antibody, denosumab, is inter alia approved for the prevention of skeletal complications in patients with bone metastases of solid tumors. Recently published studies have demonstrated its potential risk for severe osteonecrosis of the jaw, too [8–10]. Also the angiogenesis inhibitor, bevacizumab, in combination with bisphosphonates increases the risk for osteonecrosis of the jaw [11–13]. Other drugs that are associated with MRONJ are receptor tyrosine kinase inhibitors, such as sunitinib [8,14,15] and, recently, mTOR inhibitors, such as everolimus [16,17]. Depending on the different stages of MRONJ (Table 1), there are varying degrees of impairment to the patient's health and quality of life.

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Table 1
Stages of MRONJ by the American Association of Oral and Maxillofacial Surgeons (AAOMS) 2014.

Stage	*Clinical symptoms*
Risk	Preceding or existing antiresorptive medication
0	No exposed necrotic bone, but e.g. pain and/or loosening of teeth and/or intra-oral fistula
1	Asymptomatic intraoral exposed bone
2	Stage 1 plus pain and / or infection
3	Stage 2 plus extraoral fistula, pathologic fracture, oro-antral fistula

There may be a reduction in masticatory function or even the loss of tooth-wearing parts of the jaw leading to a disturbing deficit of swallowing and speech function. Because of the current lack of causal therapies, surgical treatment with removal of necrotic bone and the plastic closure is state of the art. Wound healing is often disturbed and the appearance of new areas of necrotic bone or dehisced wounds is rampant. A secondary problem is bacterial contamination of the exposed bone. In case of exposed bone bacterial superinfection is quite common. For example, in a recent review Hinson and coworkers found a contamination of the bone with *Actinomyces* in 69% of cases [18]. In addition to the antibiotic regimens, a variety of therapeutic approaches to support surgical or non-surgical therapy have been evaluated: hyperbaric oxygen therapy [19,20], platelet-rich plasma [21,22], low-level laser irradiation [23,24], vitamin D and parathyroid hormone [25,26] and bone morphogenic protein [27]. These new approaches are still under investigation.

A new and promising therapeutic method, which previously had not been applied with respect to the problem of MRONJ, is physical cold atmospheric pressure plasma (CAP). CAP offers new therapeutic options for its combined physical and biological effects (ultraviolet light, infrared light and reactive species) and shows hopeful results in the treatment of various skin and soft tissue infections [28–30]. An important aspect in the spectrum of this application is its full effect on multidrug-resistant bacteria [31,32]. For this reasons, we investigated the effect of CAP compared to chlorhexidine and control on bacterially-contaminated cancellous porcine bone with a new in vitro method of a 3-dimensional model of bacterial bone infection. We hypothesized that CFU (colony forming units) count would reveal the lowest numbers after CAP application, compared to chlorhexidine and control, and that its efficacy would exceed the bone surface.

2. Materials and methods

2.1. Bacteria cultivation

Initially, cultivation of *Streptococcus mitis* (DSM strain 12643) was performed on solid media using Columbia agar plates (Sifin, Berlin, Germany) for 24 h at 37 °C under anaerobic conditions. We choose *S. mitis* for acting as a role model in our experiments, because he is commensal in the oral cavity, acts as a initiatior of bacterial biofilm formation and shows easy culture conditions [33]. Brain-heart-infusion (BHI) broth (Sifin, Berlin, Germany) was supplemented with 5 g/L yeast extract (Yeast Extract Servabacter, Serva Electrophoresis, Heidelberg, Germany) and 1 g/L L-cysteine (Sigma Aldrich, St. Louis, MO, USA) before autoclaving and 100 mg/L hemin as well as 100 mg/L vitamin K afterward. Liquid cultures were subsequently obtained by stepwise inoculation of BHI broth with *S. mitis* until photometrically measured (Novaspec II Visible Spectrophotometer, GE Healthcare, Solingen, Germany) optical density reached 1.0 at 600 nm. Bacteria cultures were stored at 37 °C in anaerobic jars equipped with a gas generating system (Oxoid AnaeroGen, Thermo Fisher Scientific, Waltham, MA, USA). Anaerobic conditions were surveyed using oxygen indicator stripes (Oxoid Anaerobic Indicator, Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Sample preparation

Cylindrically-shaped, 4 mm diameter, xenogenetic, cortico-cancellous bone specimens (D2 quality, according to Misch et al. [34]) were retrieved from seven anterior median mandibles of four, seven-month-old commercially slaughtered pigs, using a trephan drill at 800 rpm and constant irrigation with 1% sodium chloride (NaCl). All specimens were placed into 100 ml 30% H_2O_2 for 18 h to remove the bone marrow. Afterwards, the cylinders were washed with 50 ml of 1% NaCl for 120 s and sawed into 3 mm pieces, finally obtaining 72 bone cylinders. After autoclaving, all specimens were incubated with 30 ml *S. mitis* cultures for 24 h at 37 °C under anaerobic conditions to form a biofilm. Three resin blocks (Technovit 4071, Heraeus Kulzer, Wehrheim, Germany) of 1.5 × 5 × 8 cm were created and pierced eightfold with a 3 mm drill, each hole spaced 10 mm from the next to gain slots for subsequent treatment of the specimens. Three randomly selected bone cylinders were placed in batches above each other into every slot, so only one plane surface of the cylinder on top was left exposed. A sample treatment was carried out with the specimen-loaded blocks, according to randomized assignment into groups 1–3.

2.3. Sample processing

C: control treatment with 5 ml 1% NaCl for 60 s

CHX: treatment with 5 ml 0.2% chlorhexidine (CHX; Chlorhexamed Forte, GlaxoSmithKline, Bühl, Germany) for 60 s

CAP: treatment with plasma jet (kinpen MED, Neoplas Tools, Greifswald, Germany) at 4.3 bar/argon gas flow of 4.3 slm for 60 s

Plasma jet application time was set according to the manufacturer's instructions for optimal effect on bacteria and the respective specimen's dimensions and performed statically on each sample's center at a distance of 8 mm (Fig.1). CHX volume and administration time were also set according to the manufacturer´s instructions for use.

2.4. Treatment

Rinsing agents were constantly administered using disposable syringes (B Brain Inject 5 ml, B. Braun, Melsungen, Germany) with blunt canules (0.92 × 23 mm yellow, Transcodent, Kiel, Germany).

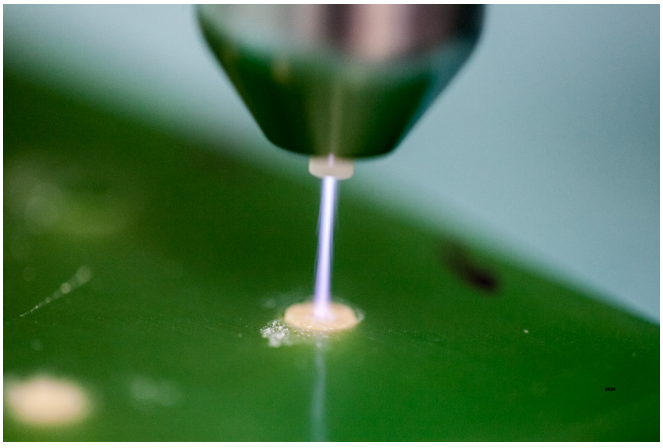

Fig. 1. Surface plasma irradiation of porcine bone cylinder.