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Effect of cerium chloride application on fibroblast and osteoblast proliferation and differentiation

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ABSTRACT

Objective: This study investigated effects of cerium-chloride on fibroblast and osteoblast differentiation and proliferation.

Methods: MC3T3-E1 cells were plated for an alkaline phosphatase (ALP) activity test. On day 3, $CeCl_3$-solutions (1, 5 or 10, w/v%) were added. After 10 s, the solutions were aspirated and washed to remove residual $CeCl_3$. On day 6 ALP activity was determined. Cell activity and proliferation was assessed by thiazolyl blue tetrazolium dye reduction assay (MTT-test) also 3 days after exposure to the $CeCl_3$-solutions. Calcium deposition by preosteoblastic cells was determined 4 weeks after the exposure of the cells by alizarin red staining. Furthermore, in all experiments the influence of adding rhBMP-2 was tested. Statistical analysis was performed by repeated-measures ANOVA using the post hoc Fisher least significant difference (LSD) test. Statistical significance was set at $p < 0.05$.

Results: Exposure to a Ce-solution of 1% or higher reduced ALP activity significantly. The addition of rhBMP-2 was able to elevate ALP activity above control level. MTT-test showed a significant decrease in cellular activity by 5% Ce or higher. The addition of rhBMP-2 had no positive effect. For human foreskin fibroblasts, exposure to even 10% Ce yielded a significant increase in cellular activity. Ce reduced calcium deposition to a level of below 50% of the control. The addition of rhBMP-2 restored mineral deposition to control levels for all Ce concentrations.

Conclusion: $CeCl_3$ had a stimulating effect on fibroblasts but a depressing influence on osteoblasts. However, adding rhBMP-2 could compensate the latter influence.

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1. Introduction

In dentistry, cerium is mainly known to improve material characteristics of titanium and zirconia by adding it into Te–Fe–Mo–Mn–Nb–Zr alloy. This results in a refinement of the crystal structure and improvement of the comprehensive mechanical properties.[1] Ceria-stabilized tetragonal zirconia/alumina (Ce-TZP/A) nanocomposites also show superior mechanical properties with significantly higher fracture toughness.[2,3]

Cerium is a member of the lanthanide series or rare earth elements, which exert diverse biological effects mainly by their resemblance to calcium. This similarity, which is particularly characteristic for the lighter members of the lanthanide series, enables these elements to replace calcium in biomolecules without necessarily substituting for its functionally.[4] Historically, cerium oxalate was used as an antiemetic, especially in vomiting of pregnancy and kinetoses, although its mechanism of action has never been clarified.[4] Cerium nitrate had a revival in the topical treatment of extensive burns exhibiting antiseptic

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effects.[5,6] The use of this compound has also gained new attention in the field of dentistry in both, preventive and therapeutic applications and strategies. In the early seventies, Mühlemann was the first to show that cerium nitrate had also the potential to reduce the solubility of rat enamel.[7] Zhang et al. tested the use of different lanthanide solutions and combinations of lanthanides with sodium fluoride, amongst them cerium, for the prevention of carious-like lesion development on root cementum and showed that the protective effects of the different lanthanide solutions.[8] Recently, Wegehaupt et al. were able to confirm these results on dentine.[9]

Nevertheless, for any biomaterial applied in contact or close to human tissues, cytocompatibility of the materials depends not only on their physical and chemical surface properties but also on the initial response of the cells on the material surface. In that context, there is a persistent debate about the cell toxicity of rare earth elements.[10,11] Although lanthanides, including cerium, have a low toxicity rating especially when they are present in materials with a low aqueous solubility, soluble cerium has been implicated in the pathogenesis of cardiac disorders such as acute myocardial infarction and endomyocardial fibrosis.[12,13]

Since any topical application in the oral cavity – even if aimed to be restricted to the non-vital outer dental hard tissues – results in potential direct contact to adjacent soft and hard tissues, effects on fibroblasts and osteoblasts are of special interest. There is limited information regarding the cell response in both cell types when applying cerium, e.g. in form of cerium chloride. Nair et al. studied the fibroproliferative response of rat heart and lung fibroblasts to cerium exposition and found that low levels of cerium (0.5 μM) stimulated a mitogenic response in cardiac fibroblasts, but the pulmonary fibroblasts were not sensitized by the element.[14] Zhang et al. demonstrated that cerium infuences the proliferation, differentiation, adipocytic transdifferentiation and mineralization function of primary osteoblasts depending on the concentration and culture time.[15]

This study was the first to assess the effects of cerium chloride on the differentiation and proliferation of human foreskin fibroblasts (HFF) and osteogenic MC3T3-E1 cells. In addition, the application of the recombinant human growth and differentiation factor bone morphogenetic protein-2 (rhBMP-2) was studied to assess the compensating and stimulating effects on the latter cells and its influence on their differentiation and maturation towards the osteogenic lineage.

2. Materials and methods

2.1. *Cerium solutions*

Preparation of 10% (w/v) $CeCl_3$ stock solution: 10 g $CeCl_3$ (Sigma–Aldrich, Buchs, Switzerland) were dissolved in 100 ml (endvolume) of bidestilled water and sterile filtered, yielding a final concentration of 268 mM. $CeCl_3$ solutions of 1% and 5% were prepared by dilution with sterile bidestilled water. Complexometric EDTA analysis revealed the Ce^{3+} fraction to be 37.6% yielding an effective Ce^{3+} of 100.91 mM for the 10% solution and 50.45 mM and 10.09 mM for the 5% and 1% solution respectively.

2.2. *Cell line, culture technique and alkaline phosphatase determination*

Human foreskin fibroblasts (HFF) were a gift from Heike Hall (ETH Zurich, Switzerland) and grown in a Dulbecco's Modified Eagle Medium (Invitrogen/Gibco, Zug, Switzerland) containing 10% foetal calf serum, 50 μg/ml penicillin/streptavidin and 1 g/l glucose. Murine preosteoblastic MC3T3-E1 cells of passage below 20 were supplied by ECACC (Porton Down, Salisbury, Great Britain) and grown in an alpha-modified Minimum Essential Medium (Invitrogen/Gibco) containing 10% foetal calf serum, 50 μg/ml penicillin/streptavidin, and 50 μg/ml ascorbic acid. For rhBMP-2 treatment, the culture medium was supplemented with 0.5 μg/ml rhBMP-2 produced in our own laboratory as described in details elsewhere.[16] To examine the biological effect, 1×10^5 cells were plated in each well of 6-well plates and grown to confluency for 2 days. On day 3 the cell culture medium was aspirated and 1 ml $CeCl_3$ solutions in phosphate buffered saline (PBS) added, either as 1, 5 or 10 (w/v) % in PBS. After an exposure time of 10 s, the respective solution was aspirated and the cells washed twice with 4 ml of PBS to remove all residual test solution before 4 ml of culture medium were added. Medium exchange was performed on day 4 and alkaline phosphatase (ALP) determined on day 6. The cells were washed 3 times with phosphate buffered saline, and the cells from a single well were combined in 0.5 ml of lysis buffer (0.56 M 2-amino-2-methyl-propane-1-ol pH 10) and homogenized using an omni-mixer. Two-hundred μl of the cell lysate were mixed with 200 μl of lysis buffer supplemented with 20 mM p-nitrophenylphosphate and 4 mM $MgCl_2$ at 4 °C, allowing the determination of ALP activity. ALP activity was normalized to total protein and expressed as nmol nitrophenylate generated per min per mg protein. This value was then used to relate it to the respective control value and displayed in percent.

2.3. *Cell proliferation*

MC3T3 or HFF cell proliferation was assessed using the thiazolyl blue tetrazolium (MTT; Sigma–Aldrich) dye reduction assay. Briefly, MC3T3 cells were grown in 6 well plates as described for ALP activity measurements. At selected time point (3 days after exposure to the respective $CeCl_3$ solutions), MTT (500 μl; 5 mg/ml in phosphate buffered saline) was added to each well and incubated for 4 h at 37 °C in the dark. Following incubation, MTT was aspirated from each well and MTT-formazan crystals were solubilised by the addition of isopropanol (200 μl; 1 N HCl). Absorbance of each well was then measured at 570 nm.

2.4. *Calcium deposition by preosteoblastic cells*

MC3T3-E1 cells were plated at a density of 30,000 cells/cm^2 in 6 well plates. Exposure to antiseptics was performed as described before and from day 3 on the culture medium was supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate. Medium was changed once a week and calcium deposition determined 4 weeks after the exposure of the cells to the antiseptics. For Alizarin red staining of calcium, culture cells were washed once with phosphate buffered saline and fixed for 1 h in 70% ethyl alcohol. After rinsing the

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