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Application of Various Cytotoxicity Assays for the Initial Evaluation of Biocompatibility of a Sr-modified dicalcium phosphate dihydrate

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The aim of the present study was to evaluate the effect of strontium-modified dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$, brushite) on viability and proliferation of cultured human Lep 3 cells using various cytotoxicity assays: thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR), crystal violet staining (CVS) and double staining with acridine orange and propidium iodide (AO/PI). The results obtained reveal high survival rate of the cells after 72 h cultivation in a $CaHPO_4.2H_2O$-modified medium – 81.5% ± 3.4 (CVS), 98.3% ± 4.6 (MTT) and 110.3% ± 4.6 (NR). No cytopathological changes were observed in the treated cells after staining with AO/PI.

Key word: Sr-modified dicalcium phosphate, cytotoxicity assays, cell culture, Lep 3 cell line, bone implants

Introduction

Bone disease is a serious health condition that directly impacts on the quality of life of sufferers. Diseases such as arthritis, tumours, and trauma can lead to defects in the skeleton requiring an operation to replace or restore the lost bone. As the population ages, the number of operations performed on bone is expected to increase (11). Bone is a tissue with a strong regenerative potential but when an area of damaged bone is too large for self-repair, the damaged bones must be repaired by using alternative materials, such as autografts, allografts and artificial materials. Bone harvested from donor sites is the gold standard for this procedure but there are limitations and complications from the use of autograft, including the limited quantity and associated chronic donor site pain. Allografts, which are transferred from other people, have problems related to not only limited availability but also with foreign body reactions and infections. As a result there is an impetus for the development of artificial bone substitute materials that do

not damage healthy tissue, do not pose any viral or bacterial risk to patients, and can be supplied at any time, in any amount (7, 8, 9, 10).

Calcium phosphate materials are widely used in surgery and dental medicine as replacements for hips, knees, teeth, tendons and ligaments, as well as repair for periodontal disease, maxillofacial reconstruction, augmentation and for stabilization of the jawbone, spinal fusion and bone fillers after tumor surgery (2). The ion-modified calcium phosphates possess some specific biologically important characteristics. For instance, it has been established that stroncium (Sr) activates osteoblats but decreases the number of osteoclasts, thus abolishing bone resorption and enhancing formation (15). The aim of the study presented was to evaluate the influence of a newly synthesized Sr-modified dicalcium phosphate dihydrate (Sr-DCP) on viability and proliferation of cultured human Lep 3 cells.

Materials and methods

Chemicals and materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (UK); dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide, neutral red, crystal violet, trypan blue, acridine orange, propidium iodide and trypsin were obtained from AppliChem (Germany). The other chemicals of the highest purity commercially available were purchased from local agents and distributors. All plasticware and syringe filters (0.2 μm) were from Orange Scientific (Belgium).

Sample preparation

Sr-dicalcium phosphate ($CaHPO_4.2H_2O$, brushite) was prepared by the biomimetic method (13, 14) at continuous co-precipitation in aqueous solution with pH 4.7 and room temperature. The modifications of the popular conventional simulated body fluid (SBFc) (6) were used as solvents for K_2HPO_4 and for the mixture of $CaCl_2$ and $SrCl_2$ in order to ensure electrolyte medium similar to blood plasma. The molar ratios in the initial solutions were (Ca+Sr)/P = 1.67 and Sr/(Sr+Ca) = 0.2. The solutions were added to precipitate with a rate of 4 ml/min. The precipitant was water washed (solid:water = 1:600), filtrated and dried at 75°C for 24 hours. Analytical reagents A.R. were used in the both synthesis.

For biological experiments 100 mg of the compound was mixed with 0.33 ml distilled water and placed on glass slide (5 cm^2) in petri dish (10 cm in diameter). After incubation for 30 min at room temperature 10 ml DMEM medium containing 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) was added to the petri dish and incubated for 4 h at 37°C. Then the medium (so called calcium phosphate modified medium, Sr-CPM-medium) was filtered twice: with a paper filter (FILTRAK) and then a syringe filter (0.2 μm). This Sr-CPM-medium was used in the biological experiments.

Experimental design

The permanent cell line Lep 3 (Cell Culture Collection, IEMPAM – BAS), established from a 3-month old human embryo, was cultured in DMEM medium, supplemented with 5-10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained at 37 °C in a humidified CO_2 incubator. The exponentially growing cells

were seeded in 96-well flat-botommed microplates at a concentration of 1×10^4 cells/well. On the 24 h the culture medium was removed and changed with Sr-CPM-medium. The biocompatibility investigations were performed using thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR), crystal violet staining (CVS) double staining method with acridine orange (AO) and propidium iodide (PI) as it was previously described (1). The data are presented as mean ± standard error of the mean. Statistical differences between control and treated groups were assessed using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test.

Results and Discussion

The experimental data obtained by MTT, NR and CVS assays are summarized in Fig. 1. The percent of viable human Lep 3 cells cultured for 72 h in the presence of Sr-CPM-medium has been found to be > 80% (81.5% ± 3.4, CVS), with no changes as compared to the control (98.3% ± 4.6, MTT) and > 100% (110.3% ± 4.6, NR). The variations observed could be explained by the different nature of the methods applied – MTT reflects damage to mitochondria), NR indicates damage to lysosomes and Golgi apparatus whereas CVS shows the growth rate reduction reflected by the colorimetric determination of the stained cells. The combined staining with acridine otrange and propidium iodide did not reveal any cytopathological changes in the cells after 72 h treatment period (Fig. 2).

Calcium phosphate materials have been found to be osteoconductive, and they can be made osteoinductive with the appropriate morphology, specifically the macroporos-

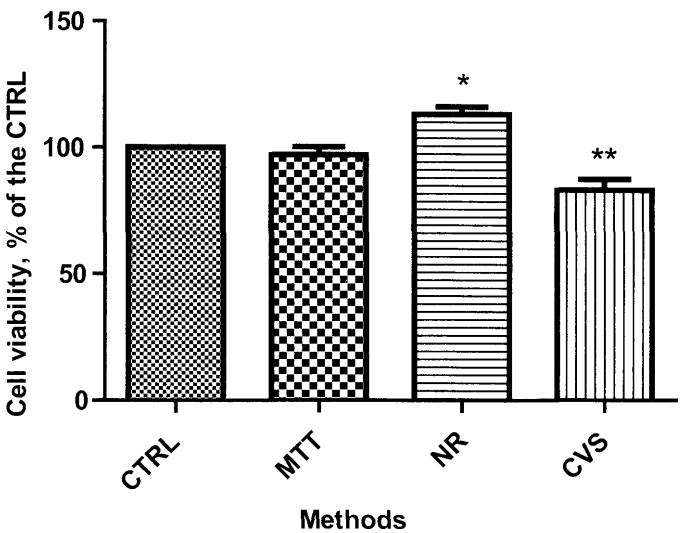

Fig. 1. Effect of Sr-modified dicalcium phosphate medium on viability and proliferation of Lep 3 human cells. The evaluation was performed by thiazolyl blue tetrazolium bromide (MTT) test; neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CVS) after 72 h treatment period. Cell viability is expressed as a percent of the untreated control (CTRL). * – $p < 0.05$ ** – $p < 0.01$

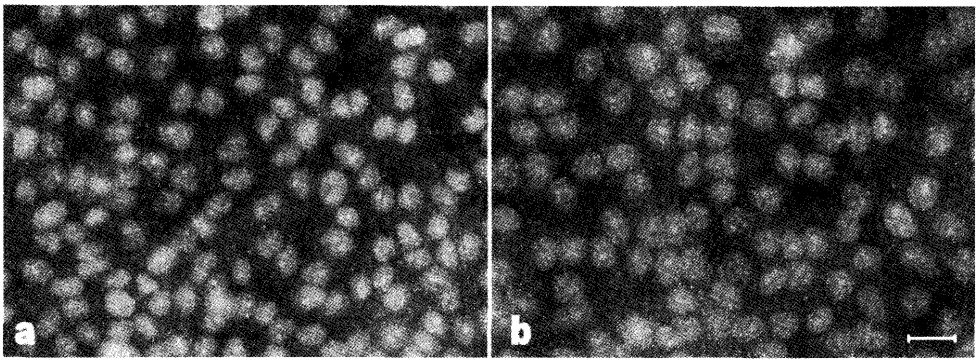

Fig 2. Untreated (Control) human Lep 3 cells (A) and cells cultured for 72 h in a Sr-modified dicalcium phosphate medium (B)

ity and microporosity (3, 4, 5, 12). It has been found in our previous investigations that Sr-modified dicalcium phosphate dehydrate, tested in the present study, does not decrease the number of viable murine fibroblasts (BALB/c 3T3) and cultured cells from bone explants (Data in press). Additional experiments are underway to clarify whether this compound will be suitable for biomedical purposes.

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