RESEARCH ARTICLE

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Influence of a Sr-modified dicalcium phosphate on viability and proliferation of murine fibroblasts and cultures from bone explants

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ABSTRACT
The aim of the study presented was to evaluate the influence of a newly synthesized strontium-modified dicalcium phosphate dihydrate (DCPD) ($CaHPO_4.2H_2O$, brushite) on viability and proliferation of murine fibroblasts (BALB/c 3T3) and cultures from bone explants. The investigations were performed by thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CVS). The cells were cultured in DMEM medium incubated for 4 h in the presence of the examined calcium phosphate placed on glass slide (100 mg compound/5 cm^2 glass slide, 10 ml medium). The results obtained revealed a high survival rate of the cells after 72 h treatment period. A positive correlation between the data coming from cytotoxicity assays with different cellular targets and mechanisms of action was observed in BALB/c 3T3 cells - NR (100.4% ± 5.5), CVS (113.4% ± 5.2) and MTT (120.5% 11.1). In addition, very close results were obtained by MTT test for BALB/c 3T3 fibroblasts (120.5% ± 11.1) and cultures from bone explants (116.7% ± 5.6). The luck of toxicity for the cells used as model systems in our study indicates that the tested material is a promising candidate for further in vivo investigations.

Key words: Sr-modified dicalcium phosphate, murine fibroblasts, bone explants, cytotoxicity assays, cell cultures

Introduction

Bone loss due to trauma or disease is an increasingly serious health problem. It has been predicted that the percentage of persons over 50 years of age affected by bone diseases will double by 2020 (Navarro et al., 2008). Bone and joint degenerative and inflammatory problems, bone fractures, low back pain, osteoporosis, scoliosis ant other musculoskeletal problems need to be solved by using permanent, temporary or biodegradable devices (Kokuko et al., 2003; Guehennec et al., 2004).

Calcium phosphate bioactive materials (ceramics, cements) have been used in the medicine and dentistry due to their chemical and structural similarity with the bone tissue mineral composition. They have such compositional resemblance to bone mineral that they induce a biological response similar to the one generated during bone remodeling. Thus, during resorption, the degradation products of calcium phosphate bioceramics (calcium and phosphate ions) are naturally metabolized and they do not induce abnormal calcium or phosphate levels in urine, serum, or organs (liver, skin, brain, heart, kidney, lung, and intestine) (den Hollander et al., 1991; Qin et al., 2004; Peacock, 2010).

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 (Zofková et al. 1997).

The aim of the study presented was to evaluate the influence of a newly synthesized Sr-modified dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$, brushite) on viability and proliferation of murine fibroblasts (BALB/c 3T3) and cultures from bone explants.

Materials and Methods

Chemicals

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 (MTT), neutral red, crystal violet, trypan blue and trypsin were from AppliChem (Germany). The enzyme accutase was a generous gift from IVD, Bulgaria. The other chemicals of the highest purity commercially available were purchased from local agents and distributors. The plasticware and syringe filters (0.2 µm) were from Orange Scientific (Belgium).

Sample preparation

Sr-modified dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$, brushite) was prepared by the biomimetic method (Rabadjieva et al. 2011a, 2011b) at continuous co-precipitation in aqueous solution with pH 4.7 and room temperature. The modifications of the popular conventional simulated body fluid (SBFc) (Kokubo, 1990) 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, CPM-medium) was filtered twice: with a paper filter (FILTRAK) and then a syringe filter (0.2 µm). This CPM-medium was used in the biological experiments.

Experimental animals

Five ICR mice (2 month old) from both sexes were purchased from Laboratory Animal Center (Slivnitsa, Bulgaria). Animals were given standard pellet diet and tab water *ad libitum*.

The experiments with laboratory animals were performed in accordance to the guidelines of Veterinary Medical Office in Bulgaria which follow the European Committee Standards concerning the care and use of laboratory animals (Registrations 25/26.01.2011 by the Regional Veterinary Medical Office, Sofia, and 11130127 by the National Veterinary Medical Office in Bulgaria).

Cell cultures and cultivation

Murine fibroblasts

The permanent cell line BALB/c 3T3 clone 31 (mouse embryo fibroblasts) was obtained from Centro Substrati Cellulari (Brescia, Italy) (Aaronson & Todaro, 1868).

The cells were grown as monolayer culture in DMEM medium, supplemented with 5-10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The cultures were maintained at 37ºC in a humidified CO_2 incubator (Thermo Scientific, Hepa Class100). For routine passages adherent cells were detached using a mixture of 0.05% trypsin and 0.02% EDTA. The experiments were performed during the exponential phase of cell growth.

Cell cultures from bone explants

Cell cultures from femurs of ICR mice were obtained by cutting the two epiphyses and separating the diaphyse in half. Every piece of the diaphyse is placed on a drop of serum in a well of a 12-well plate. After 1 h of incubation at room temperature DMEM medium with 20% FBS and antibiotics was added carefully and the plates were left in a thermostat. Every 4-5 days in the next 21 days half of the culture medium was replaced with fresh one. On the 21st day the bone particles were removed from the wells and all the medium was replaced with fresh DMEM with 20% FBS.

After having achieved a sufficient size cell colonies were passaged to new wells using the enzyme accutase. In the next two weeks the cells were multiplied and passaged to new wells. On the third week the cells from all wells were transferred to a 25 ml flask. After two more weeks of

multiplication and passage cytotoxicity test with CPM-medium was conducted.

Cytotoxicity assays

The cells were seeded in 96-well flat-bottommed microplates at a concentration of 1×10^4 cells/well. After the cells were grown for 24 h to a subconfluent state (~ 60-70%), the cells from monolayers were washed with phosphate buffered saline (PBS, pH 7.2) and covered with media modified with CPM-medium (8 wells for each test). Samples of cells grown in non-modified medium served as controls. After 72 h of incubation, the effect of CPM-medium on cell viability and proliferation was examined by thiazolyl blue tetrazolium bromide (MTT) test (Mosman, 1983), neutral red uptake cytotoxicity assay (NR) (Borenfreund & Puerner, 1885) and crystal violet staining (CVS) (Saotome et al., 1989).

Optical density was measured at 540 nm using an automatic microplate reader (TECAN, Sunrise™, Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each cytotoxicity assay.

Statistical Analysis

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 and student t-test with use of GraphPad 5.00 program (La Jolla, CA, USA).

Results

The viability of the cells used as experimental models in our study was evaluated after 72 h culturing in the presence of CPM medium by thiazolyl blue tetrazolium bromide test, neutral red uptake cytotoxicity assay and crystal violet staining. The results obtained are summarized in Figures 1 and 2.

Discussion

The results obtained revealed a high survival rate of BALB/c 3T3 murine fibroblasts and a positive correlation between the data coming from cytotoxicity assays with different cellular targets and mechanisms of action – NR (100.4% ± 5.5), CVS (113.430% ± 5.2) and MTT (120.5% 11.1). Very close results were obtained by MTT test for 3T3 fibroblasts (120.5% ± 11.1) and cultures from bone explants (116.7% ± 5.6). The MTT test is based on the ability of viable mitochondria within cells to reduce succinic dehydrogenase; the basic NR dye distributes to the acidic compartments in the cell and therefore acts as a marker for the integrity of lysosomes and possibly of the Golgi apparatus; and the CV staining shows the growth rate reduction reflected by the colorimetric determination of the stained cells.

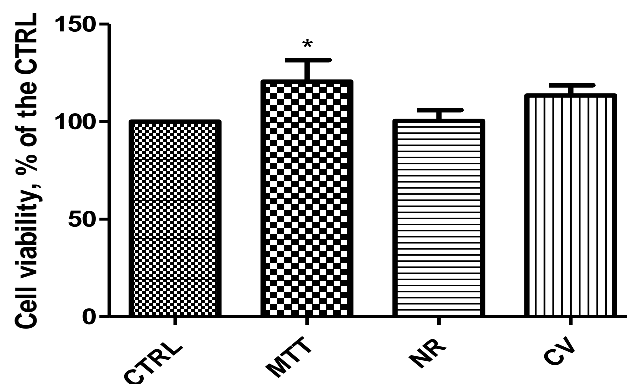

Figure 1. *Effect of a Sr-modified dicalcium phosphate dihydrate -modified medium on viability and proliferation of BALB/c 3T3 murine fibroblasts. The evaluation was performed by thiazolyl blue tetrazolium bromide (MTT) test; neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CVS) after 72 h treatment period. Cell viability is expressed as a percent of the untreated control (CTRL). * - p < 0.05.*

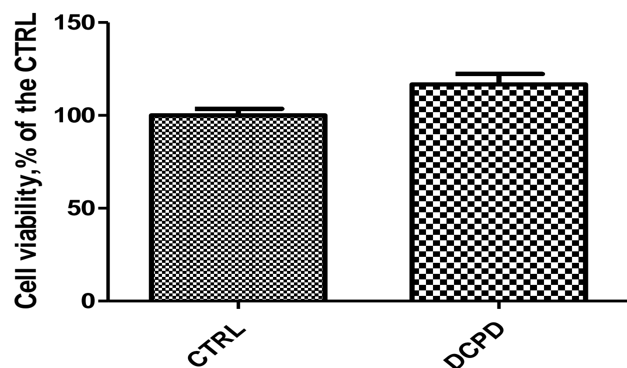

Figure 2. *Effect of a Sr-modified dicalcium phosphate dihydrate modified-medium on viability and proliferation of cultured murine cells from bone explants. The evaluation was performed by thiazolyl blue tetrazolium bromide (MTT) test after 72 h treatment period. Cell viability is expressed as a percent of the untreated control (CTRL). During MTT test the cell cultures from bone explants were on the 8th passage.*

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The decision to use BALB/c 3T3 murine fibroblasts as model system in our study was not occasional because it is well known that fibroblasts take part in bone healing process (Kalfas, 2001). On the other hand, the cells proliferating from the bone explants are suitable experimental models for evaluating the biocompatibility of calcium phosphate materials because they represent at least to some extent the situation of regenerating damaged bone in vivo. Something more, in contrast to permanent cell lines that have undergone many passages in vitro, primary cultures resemble better the biological characteristics of the cells in the tissue of their origin.

The luck of toxicity for the cells used as model systems in our study indicate that the tested material is a promising candidate for further *in vivo* investigations.

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