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Original Contribution

Mg-MODIFIED CALCIUM PHOSPHATE – A PROMISING MATERIAL FOR BONE IMPLANTS

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ABSTRACT
PURPOSE: The aim of this study was to evaluate the effect of Mg-modified tricalcium phosphate material (Mg-TCP) on viability and proliferation of MRC-5 human embryonic firbroblasts.
MATERIALS AND METHODS: The investigations were performed using thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR), crystal violet staining (CV) and trypan blue dye exclusion technique (TB).
RESULTS: The results obtained revealed that after 72 h of treatment the percent of viable MRC-5 cells cultured in the presence of Mg-TCP was 107.67 % ± 5.32 (MTT), 99.09 % ± 3.95 (NR) and 116.04 % ± 5.38 (CV). The growth potential of MRC-5 cells (seen in growth curves prepared after 1, 2, 3, 4, and 7 days treatment periods, TB) was similar to those of the untreated control cells. CONCLUSION: The results obtained by us show that the investigated Mg-modified tricalcium phosphate could be considered as a promising material for bone regeneration medicine. Additional investigations are underway to clarify better the biocompatibility of the examined ion-modified TCP material as well as its osteoconductivity and osteoinductivity.

Key words: tricalcium phosphates, magnesium, biocompatibility, human embryonic fibroblasts

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 (1). 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 (2-3).

Calcium phosphate bioactive materials (ceramics, cements) have been used in the medicine and dentistry due to their chemical and structural resemblance to bone tissue mineral composition. They induce a biological response similar to the one generated during bone remodeling. 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.

The ion-modified calcium phosphates possess some specific biologically important characteristics. Thus, Mg is among the preferable modifiers as it is an essential trace element for the organisms. Magnesium is closely associated with mineralization of calcified

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tissues, and indirectly influences mineral metabolism (4). Mg and Zn modified tricalcium phosphate ceramics were found to exhibit lower solubility than the pure ones (5-6) and hence reduce the resorption rate (7).

The aim of this study was to evaluate the effect of magnesium-modified tricalcium phosphate (Mg-TCP) on viability and proliferation of embryonic human fibroblasts MRC-5.

MATERIALS AND METHODS

Synthesis of Mg-TCP

Mg-modified tricalcium phosphate was synthesized by the method of biomimetic continuous co-precipitation and further high temperature treatment (8). The obtained powder was characterised by chemical and XRD analysis. Thus the used in this study Mg-TCP is characterised by a cationic ratio Mg/(Mg+Ca) = 0.02 .

Chemicals and other materials for the in vitro studies

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FCS) were purchased from Gibco-Invitrogen (UK); neutral red, crystal violet and trypan blue were purchased from AppliChem (Darmstadt, Germany); thiazolyl blue tetrazolium bromide (MTT) was obtained from Sigma-Aldrich Chemie GmbH (Germany). Sterile phosphate buffered saline (PBS) at pH 7.2-7.4, dimethyl sulfoxide, and all other chemicals of the highest purity commercially available were purchased from local agents and distributors. All plastic ware and syringe filters were from Orange Scientific (Belgium).

Sample preparation

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% FCS 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 Mg-TCP-medium) was filtered twice: with a paper filter (FILTRAK) and then a syringe filter (0.2 μm). This Mg-TCP medium was used in the biological experiments.

Cells and culturing

The embryonic fibroblasts MRC-5 (obtained from a 14-week old male human fetus) (9) were used as an experimental model in our study. The cells were obtained from Cell Culture Laboratory in National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria. They were grown as monolayer culture in DMEM medium, supplemented with 5-10% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin. The cultures were kept in a humidified incubator (Thermo Scientific, Hepa class 100) at 37°C under 5% CO_2 in air. 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.

Cytotoxicity assays

The cells were seeded in 96-well flat-bottomed 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 Mg-TCP medium. After 72h of incubation the cell viability was examined by thiazolyl blue tetrazolium bromide (MTT) test (10), neutral red uptake cytotoxicity assay (NR) (11) and crystal violet staining (CV) (12). Optical density was measured at 540 nm using an automatic microplate reader (TECAN, SunriseTM, Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated.

Growth curve preparation

The MRC-5 cells were seeded in Mg-TCP medium in 12-well plates. The number and viability of cells (collected from 2 wells) were determined on 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 7^{th} day by trypan blue dye exclusion technique using a Cell counter Countess ^{TM} (Invitrogen).

Statistical analysis

The data are presented as mean ± standard error of the mean. Statistical analysis was assessed by unpaired *t*-test and use of GraphPad 5.00 Program (La Jolla, CA, USA) or Microsoft Office Excel 2007 (Microsoft Office 2007, Version 1.2).

RESULTS AND DISCUSSION

The data of MRC-5 cells viability obtained by the methods with different cell targets (MTT, NR, CV, TB) and mechanisms of action revealed that the examined Mg-TCP did not affect

significantly the viability and proliferation of cultured MRC-5 human embryonic fibroblasts **(Figure 1 and 2)**: the percent of viable cells was found to be 107.67% ± 5.32 (MTT), 99.09% ± 3.95 (NR) and 116.04% ± 5.38 (CV) as compared to the untreated control. The growth potential of MRC-5 cells cultured in Mg-TCP medium was very close to those of the control cells **(Figure 2)**, for example their viability examined by trypan blue dye exclusion technique was 92.31 % ± 2.69 on the 7th day.

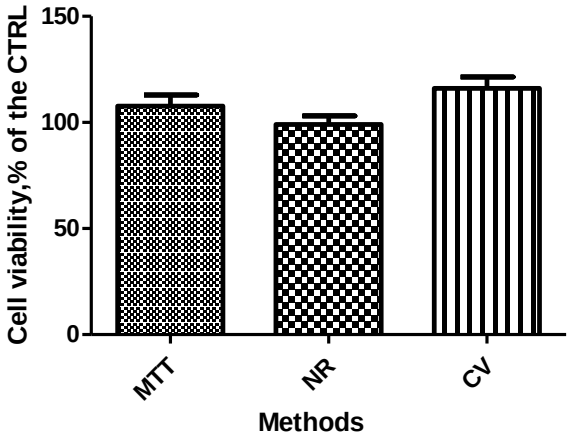

Figure 1. Effect of Mg-modified tricalcium phosphate (Mg-TCP) on viability and proliferation of cultured MRC-5 human embryonic fibroblasts. The evaluation was performed after 72 h of treatment by thiazolyl blue tetrazolium bromide test (MTT), neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CV).

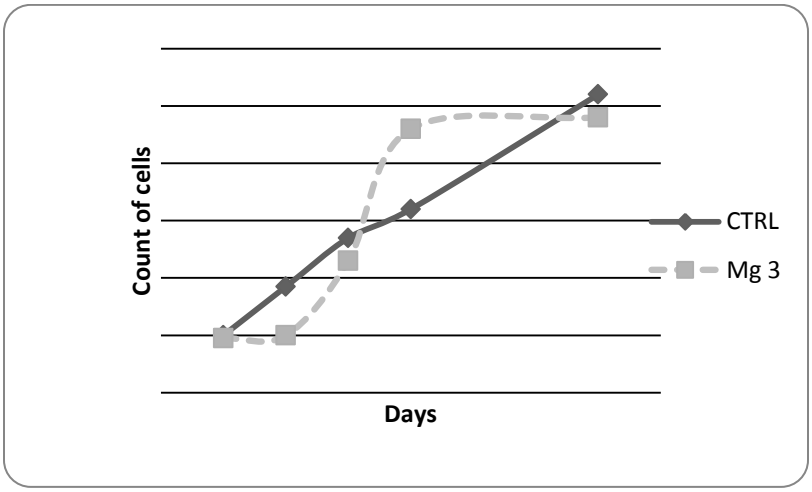

Figure 2. Growth curve of MRC-5 human embryonic fibroblasts cultured in Mg-TCP medium. The cell number and viability were determined by trypan blue dye exclusion technique using Automated Cell counter Countess ™ (Invitrogen).

The decision to use MRC-5 cells as model system in our study was not occasional because these are normal diploid human cells exhibiting fibroblast morphology (9) and it is well known that fibroblasts take part in bone healing process (13). It has been found in our previous investigations that after 72 h of treatment the percent of viable murine bone marrow cells cultured in the presence of the same Mg-TCP

was 63.11% ± 1.10 (MTT test) and 46.445% ± 1.29 (TB technique) whereas the viability of primary cultures from murine bone explants was 97.8 % ± 7.2 (72 h, MTT) (14). The different sensitivity of the cells (permanent cell lines and primary cultures, human and mouse; bone

marrow, fibroblasts) used as model systems in our experiments could be explained by cell specific response. The lowest rates of viability and proliferation were found for murine bone marrow cells. It is well known that because of a high proliferation rate and lack or very low expression of the mdr1 gene (coding for P-glycoprotein that is responsible for the efflux of xenobiotics out of the cell) usually the bone narrow cells are very sensitive to the influence of xenobiotics (15).

Due to their compositional similarities to bone mineral and excellent biocompatibility, calcium phosphate biomaterials are successfully applied in cranio-maxillofacial, dental, and orthopedic surgery and are the most widely used bone substitutes in bone tissue engineering. Something more, in recent years, tricalcium phosphate materials have attracted significant interest in simultaneous use as bone substitute and vehicles for growth factors and drugs in bone tissue engineering, adding a new dimension to their application (16). The results obtained by us show that the investigated Mg-modified tricalcium phosphate could be considered as a promising material for bone regeneration medicine. Additional investigations are underway to clarify better the biocompatibility of the examined ion-modified TCP material as well as its osteoconductivity and osteoinductivity.

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