VERSITA

Biologia **63**/2: 273—281, 2008
Section Cellular and Molecular Biology
DOI: 10.2478/s11756-008-0043-x

Comparative study of a new composite biomaterial fluor-hydroxyapatite on fibroblast cell line NIH-3T3 by direct test

Marica Theiszová[1], Soňa Jantová[1], Silvia Letašiová[1], Ľuboš Valík[1] & Martin T. Palou[2]

[1]*Institute of Biochemistry, Nutrition and Health Protection, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK-81237 Bratislava, Slovakia; e-mail: marica.theiszova@stuba.sk*
[2]*Institute of Inorganic Chemistry, Technology and Materials, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK-81237 Bratislava, Slovakia*

Abstract: The number of biomaterials used in biomedical applications has rapidly increased in the past two decades. Fluorapatite (FA) is one of the inorganic constituents of bone or teeth used for hard tissue repairs and replacements. Fluor-hydroxyapatite (FHA) is a new synthetically prepared composite that in its structure contains the same molecular concentration of OH^- groups and F^- ions. The aim of this experimental investigation was to use the embryonal mouse fibroblast cell line NIH-3T3 for comparative study of basal cytotoxicity of fluoridated biomaterials FHA and FA discs. Hydroxyapatite (HA) disc, high-density polyethylene as negative control and polyvinyl chloride (PVC) containing organotin stabilizer as positive control were used as standard biomaterials. The appropriateness of the use of NIH-3T3 cells and their sensitivity for tested biomaterials were evaluated on the basis of five cytotoxic end points: cell proliferation, cell morphology, lactate dehydrogenase (LDH) released, protein and DNA cell content. The basal cytotoxicity of FHA, FA and HA discs was measured by direct contact method. FHA composite, FA and HA demonstrated in cell line NIH-3T3 nearly similar basal cytotoxicity increasing with the time of treatment. After 72 h of biomaterials treatment, about 25% inhibition of cell number, unchanged morphology of dividing cells, 6.31–0.16% increase of released LDH, about 10% inhibition of cell protein content and about 20% inhibition of DNA content was found. On the other hand, from the growth rates it resulted that NIH-3T3 cells, affected by tested biomaterials, divided about 20% slowlier than the control (untreated cells). Using the linear regression analysis we found out that deviations in measurements of cytotoxicity by four methods were as follows: less than 10% for cell number, protein and DNA content methods and 12.4% for released LDH method. Based on a good correlation of the cytotoxicity of biomaterials obtained from all end points we could conclude that fibroblast NIH-3T3 cell line was appropriate for measuring the basal cytoxicity of tested biomaterials.

Key words: fluor-hydroxyapatite composite; fluorapatite; hydroxyapatite; NIH-3T3 fibroblasts; contact basal cytotoxicity.

Abbreviations: FA, fluorapatite; FHA, fluor-hydroxyapatite; HA, hydroxyapatite; LDH, lactate dehydrogenase; PVC, polyvinyl chloride.

Introduction

There is an increasing need for medical implants, due to an increasing aged population. Bone defects resulting from trauma; disease or developmental anomalies can substantially be improved by reconstructive surgery. Small improvements in the design and constituent materials of such implants can greatly reduce the healing time and the need for expensive, traumatic revision surgery. Commonly used bioactive coatings, bioactive ceramics, bioactive glass or glass-ceramics, bio-inactive ceramics, calcium phosphates and different composite biomaterials reduce healing time but are eventually absorbed by the body, leaving the substrate bare.

Bone is essentially constituted of nanoscale inorganic materials and proteins. The inorganic materials are minerals which are structurally apatite-like, such as hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$], fluorapatite [FA, $Ca_{10}(PO_4)_6F_2$] and carbonate-apatite (Gineste et al. 1999).

HA is considered as the most promising materials for hard tissue repairs and replacements due to its similar chemical composition and crystallographic structure to that of bone mineral. It has therefore been studied extensively and prepared for clinical applications. HA has also attracted much attention for use as a substitute for teeth due to its excellent biocompatibility to human tissues. Nevertheless, HA intrinsic poor mechanical properties (strength, toughness and hardness) have restricted wider applications in load-bearing implants. Therefore, composite HA materials have been developed by different synthesis routes and techniques in order to improve both bioactivity and mechanical properties of various orthopaedic prosthesis and dental implants. Bioactive ceramics, bioactive glass or glass-ceramics, bio-inactive ceramics, polymers and metals have all been used to fabricate HA composites (Ning &

Dai 2003). Many of them have demonstrated an excellent biocompatibility and ideal bioactivity both *in vitro* and *in vivo* tests (Jarcho et al. 1977; Rejda et al. 1977; LeGeros 1993; LeGeros et al. 2003).

Beside HA, FA or fluorided hydroxyapatite is a widely spread form of calcium phosphate present particularly in biological material (Leroy & Bres 2001). FA is considered as biomedical material due to its structure similar to HA. Some studies in the last 10 years were extended to the synthesis of solid solution fluor-hydroxyapatite (FHA). The fluoride ion is partially or totally substituted for hydroxide in the OH^- lattice position in HA forming thus a large range of solid solutions (composites) of FHA, with a formulae of $Ca_{10}(PO_4)_6(OH)_{2-x}F_x$. The importance of such approach is related to the presence of partially fluorided HA found in bone and mainly in tooth enamel. It has been found that fluoride is uniformly distributed within the bone tissue or within the thin tooth enamel outer layer. It is clear that FHA composites are homogeneous solid solutions and not a simple mixture of HA and FA. Our recent studies (Kuzielová et al. 2006) on the synthesis and characterization of solid solutions between HA and FA have shown that FA has higher thermal stability than HA. It is therefore expected that introduction of FA will retard the decomposition of HA. In addition, the FA itself retains advantages over other ions in that it protects teeth from dental caries, especially in the environment in the oral cavity as well as enhances mineralization and crystallization. Some studies have revealed that FA has good biocompatibility *in vivo*, HA substituted with fluorine had higher osteoblastic cellular activity (Marugan et al. 2002) and micro-molar concentrations of fluorine were effective to stimulate cell proliferation and differentiation (Farley et al. 1983). This gives a good opportunity to extend the research to FHA. The composite can be used in different fields of surgery medicine as surface coatings on various orthopaedic prosthesis and dental implants.

Synthetic HA-bioceramics are obtained by different chemical and technical processes (Karen et al. 2000). They can be generally divided in two main groups: solid-state reactions and wet processes. The solid-state reactions include precipitation, hydrothermal technique and hydrolysis of other calcium phosphates (Mobasherpour et al. 2007), while the wet route concerns mainly the sol-gel process (Pereira et al. 2005; Xu et al. 2006). Depending upon the route and technique used, HA bioceramics with various morphology, Ca/P ratios, microstructure and level of crystallinity can be obtained. Therefore, various HA can be prepared for various applications (Tadashi et al. 2003). The temperature has also a great deal of influence on the particle size and morphology of precipitated HA. It was demonstrated that nano-HA provides several biological benefits over HA of larger particle size in its application in bone tissue engineering because of its higher surface area and thus higher bioactivity.

In our previous works we have prepared FHA, FA and HA by precipitation method and examined the cytotoxic, genotoxic and mutagenic activity of their eluates by indirect contact (Theiszová M., Jantová S., Letašiová S. & Palou M., unpublished results). After 72 h of treatment, cytotoxic effect obtained from cell proliferation, protein/DNA content and lactate dehydrogenase (LDH) released of all tested biomaterials to murine fibroblast NIH-3T3 cells was weak (3.1–25%). On the other hand, 5 day's eluates of FHA and FA inhibited the growth of leukemia L1210 cells and induced programmed cell death through mitochondrial/caspase-9/caspase-3-dependent pathway. The results from cytotoxic, genotoxic/mutagenic studies on V79 cells showed that the highest tested concentrations of all tested biomaterials (100 and 75% eluates) induced very weak inhibition of colony growth (about 10%). On the other hand, the inhibition of cell number per colony induced by these concentrations was in the range from 42.9% to 30.62%. Comet assay showed that biomaterials induced DNA breaks of V79 cells which increased with used concentrations in order HA<FHA<FA. All biomaterials tested towards *Salmonella typhimurium* TA100 and V79 cells did not induce mutagenic effect against positive control (N-methyl-N'-nitro-N-nitrosoguanidine) and DNA breaks was probably the reason for the inhibition of cell division in V79 cell colonies.

The aim of this experimental study was to use the embryonal mouse fibroblasts NIH-3T3 cell line for comparative study of basal cytotoxicity of fluoridated biomaterials FHA and FA discs. HA disc, high-density polyethylene as negative control and polyvinyl chloride (PVC) containing organotin stabilizer as positive control were used as standard biomaterials. The appropriateness of the use of these cells and their sensitivity to tested biomaterials were evaluated on the basis of five cytotoxic end points: cell proliferation, cell morphology, LDH released, protein and DNA cell content. The basal cytotoxicity of FHA, FA and HA discs was measured by direct contact method.

Material and methods

Biomaterials

The tested biomaterials were HA, FA, FHA composite, high-density polyethylene (Chemopetrol a.s., Czech Republic) as negative control, PVC-containing organotin stabilizer (Institute of Chemical Technology, Prague, Czech Republic) as positive control. HA disc was prepared by homogeneous precipitation method using $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ as starting materials and ammonia solution as agents for pH adjustment. Equations (1–3) illustrate chemical reactions leading to the precipitation of HA, FA and FHA, respectively. A suspension of $Ca(NO_3)_2 \cdot 4H_2O$ powder was diluted in deionised water and stirred at 25 °C. Then, a solution of $(NH_4)_2HPO_4$ was slowly added by drop-wise to the $Ca(NO_3)_2 \cdot 4H_2O$ solution. In all experiments the pH of $Ca(NO_3)_2 \cdot 4H_2O$ solution was kept by ammonia solution at 10. The final solution was stirred at room temperature for 3 h. Then, the precipitate formed was filtered off, washed with deionised distilled water several times to the neutral pH, and finally dried under infra-red lamp for 24 h. After drying, the sample was powdered and treated at 900 °C for

1 h. The composition of the obtained product was controlled by powder X-ray diffraction. HA has been identified as Hap JCPDS 9-438.

The chemical process leading to HA can be explained by the following reaction (Eq. 1):

$$10\ Ca(NO_3)_2 \cdot 4H_2O + 6\ (NH_4)_2HPO_4 + 8\ NH_4OH \rightarrow \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20\ NH_4NO_3 + 43\ H_2O$$

Likewise the FA was obtained according to the reaction (Eq. 2):

$$10\ Ca(NO_3)_2 \cdot 4H_2O + 6\ (NH_4)_2HPO_4 + 8\ NH_4F \rightarrow \rightarrow Ca_{10}(PO_4)_6F_2 + 20\ NH_4NO_3 + 6\ HF + 40\ H_2O$$

The solid solutions of FHA were prepared by precipitation method (Eq. 3):

$$10\ Ca(NO_3)_2 \cdot 4H_2O + 6\ (NH_4)_2HPO_4 + 4\ NH_4OH + + 4\ NH_4F \rightarrow Ca_{10}(PO_4)_6(OH)F + 20\ NH_4NO_3 + + 3\ HF + 43\ H_2O$$

Cell culture

NIH-3T3 fibroblast cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line NIH-3T3 grown at 37 °C in humidified 5% CO_2 and 95% air atmosphere was cultured in completely Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum, penicillin G (100 μg/mL) and streptomycin (100 μg/mL). All culture medium compounds were obtained from Biocom Company (Slovakia). Before a uniform monolayer of NIH-3T3 cells was formed, cells were freed from the surface of the culture dish by a 0.25% solution of trypsin and were sub-cultivated two-three times a week.

NIH-3T3 cultured cells were re-suspended in culture medium and the total number of cells and their viability was measured. Cell viability was determined by a Trypan blue exclusion test.

Direct contact assay

The basal cytotoxicity of positive control, negative control, FHA, HA and FA discs was determined using the method of direct cell counting (Lang & Mertens 1990). NIH-3T3 cells were re-suspended in culture medium at density of 65,000 cells/mL and plated (325,000 cells/dish) into 60 mm Petri dishes in a total volume of 5 mL medium. The dishes were incubated for 24 h, at 37 °C in a humidified atmosphere of 5% CO_2 in air. After then, the medium was replaced by the fresh medium and the positive control, negative control and tested biomaterial discs (diameter of disc 10 mm) were placed in the centre of the Petri dishes, under sterile condition. The control was performed by seeding the cell suspension in the Petri dishes without the tested biomaterial discs in the centre (Fig. 1a). Cell proliferation was evaluated after 24, 48 and 72 h in the absence or in the presence of positive control, negative control, FHA, HA and FA discs. At each time, the medium was removed, cells in monolayer were trypsinized (0.25% trypsin) (Biocom, Slovakia) for 3 min at 37 °C and counted in a Bürker chamber. Viability of the treated and control cells were determined by LDH assay. The cellular monolayer was observed with a light microscope (Meopta, Slovakia) and photographed by Panasonic DMC/FX3.

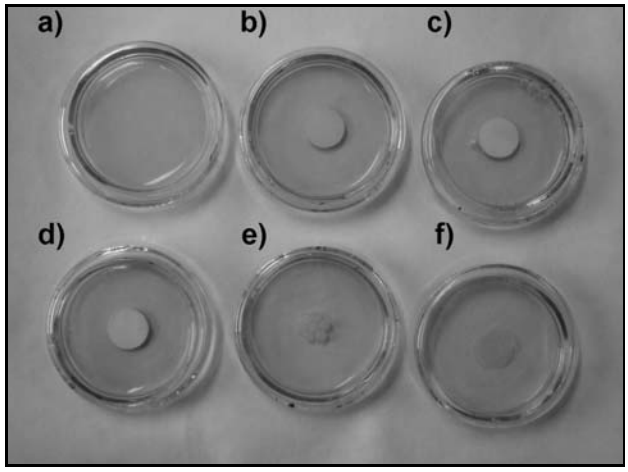

Fig. 1. Direct test for detecting the cytotoxicity effects of biomaterials on *in vitro* cell culture. The tested discs of biomaterials (diameter 10 mm) were placed in the centre of a 60 mm Petri dish. (a) Control, (b) FHA, (c) FA, (d) HA, (e) negative control (high-density polyethylene) and (f) positive control (PVC-containing organotin stabilizer).

LDH quantification

Measurement of LDH release is an important and frequently applied test for cellular membrane permeabilization and severe irreversible cell damage. The amount of released LDH was measured according to Bergmeier (1970).

After appropriate incubation periods, the same volume of the medium with and without cells was aspirated for each sample and stored on ice until measurement. Then, the standard solutions for samples with released LDH (100 mM Tris-HCl buffer, pH 7.1, 15 mM NADH, 1.0 M pyruvate sodium salt) and total LDH (the same composition as for released LDH plus 10% solution of Triton-X-100) were prepared. The standard solutions were incubated at 31 °C for 5–10 min before measurement. The enzymatic reaction started by adding sample that was very gently shaken before, into the standard solution. The oxidation of NADH was measured on PU 8750 UV/VIS scanning spectrophotometer PHILIPS at $\lambda = 340$ nm. The absorbance decreases linearly with time over 60 s of measurement.

DNA content

The DNA content of each sample was measured using the DNA Quantitation kit (Sigma, Slovakia) according to Labarca & Paigen (1980). It is a fluorimetric method based on the binding of the bis-benzimide Hoechst stain (Hoechst 33258, Sigma) to DNA.

After appropriate incubation periods, the samples were washed twice with phosphate buffer saline solution and trypsinized using 0.25% solution of trypsin. The cells were re-suspended in 1 mL of phosphate buffer saline solution. The Hoechst stain was prepared at a concentration of 1 mg/mL in 1x TNE buffer (10x TNE buffer: 100 mM Tris; 2.0 M NaCl; 10 mM EDTA; pH 7.4). Test samples (25 μL) were pipetted into a minicell cuvette. To each of these cuvettes, 50 μL of the Hoechst stain and 25 μL of 1X TNE buffer were added. The samples were incubated for 2–5 min at room temperature and protected from light. The samples were read on the Fluorescent Module of GloMax^{TM} 20/20 Luminometer (Promega, USA) at $\lambda = 360$ nm excitation and $\lambda = 460$ nm emission. The concentrations of DNA in test samples were calculated using a standard curve gener-

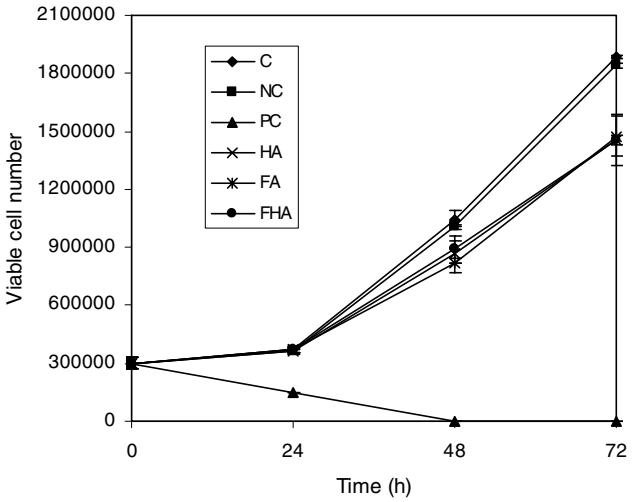

Fig. 2. Direct effect of tested biomaterials on the cell proliferation. C, control; NC, negative control (high-density polyethylene); PC, positive control (PVC-containing organotin stabilizer), HA, hydroxyapatite; FA, fluorapatite; and FHA, fluor-hydroxyapatite. Each point represents mean values ± S.D. from three independent experiments (for each type of biomaterial three separate Petri dishes were used in each experiment).

ated from the known concentrations of DNA (Calf Thymus DNA, Sigma).

Protein content

After 24 h of NIH-3T3 cell cultivation in Petri dishes (65,000 cell/mL) the positive control, negative control and tested biomaterials were added. After 24, 48 and 72 h incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, the content of total cellular proteins according to Lowry et al. (1951) was determined. Bovine serum albumin was used as a standard.

Statistical analysis

Results were shown as the arithmetic means ± S.D. of the mean of three separate experiments (for each type of biomaterial three separate Petri dishes were used in each experiment). Statistical analysis was performed with the ANOVA test for nonparametric measurements ($P<0.05$ was considered statistically significant). The collected data were analysed using linear regression tools of the Excel 2007 statistical software package (Microsoft Corp.).

Results

Direct effect of biomaterials on proliferation of NIH-3T3 cells

Figure 2 shows the direct effect of positive control, negative control and tested biomaterials FHA, FA and HA on cell proliferation evaluated at 24, 48 and 72 h of culture. While positive control (PVC-containing organotin stabilizer) induced total lysis of cell population, NIH-3T3 cells treated by negative control (high-density polyethylene) grew as control, untreated NIH-3T3 cells. FHA, FA and HA induced inhibition of NIH-3T3 cell proliferation. In the first time interval (24 h), the proliferation of cells affected by biomaterials was 94.51% for FHA, 96.34% for FA and 96.34% for HA. After 72 h incubation, the inhibition of cell division was about 25% for all tested biomaterials.

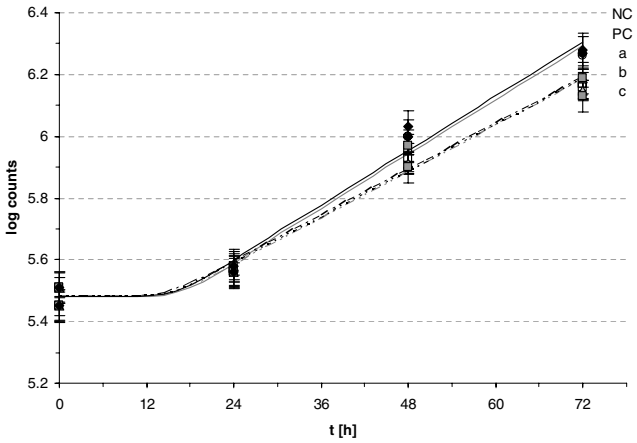

Fig. 3. Growth curve of NIH-3T3 cells treated by biomaterials and fitted by primary model of Baranyi et al. (1993). NC, negative control; PC, positive control; a, hydroxyapatite; b, fluorapatite; and c, fluor-hydroxyapatite.

Based on obtained cell numbers the primary model according to Baranyi et al. (1993) was applied for the mutual comparison of cytotoxicity of biomaterials and the relevant growth rates of cell proliferation was calculated (Fig. 3). It was found out that while control cells divided by the rate of 0.015 log/h (0.36 log/day), the cells affected by tested biomaterials proliferated by the rate of 0.012 log/h (0.29 log/day).

Direct effect of biomaterials on morphology of NIH-3T3 cells

NIH-3T3 cells were plated in 60 mm Petri dishes either in the presence or in the absence of the positive control, negative control and tested biomaterials. The microscopic observations and the photos were carried out starting from the centre to the periphery of the dishes where chemicals located. NIH-3T3 cells treated with positive control were degenerated and lysed (Fig. 4). On the other hand, control cells and the cells treated with negative control, FHA, FA and HA were homogeneously distributed on the substrate and produced a complete monolayer after 72 h of culture. The great majority of them were scattered and exhibited typical fibroblast morphology (an elongated and polygonal shape). In some areas, cells in mitosis were observed. NIH-3T3 cells grown in direct contact with FHA, HA and FA (Fig. 4) did not show any morphological damage at 24, 48 and 72 h of culture. Their morphology was completely similar to that of control cells.

Direct effect of biomaterials on viability of NIH-3T3

To study the cell viability, negative control and discs of FHA, FA and HA were incubated with murine fibroblast cell line NIH-3T3. Extra-cellular LDH concentrations were measured and compared between control cells and cells cultured with negative control, FHA, FA and HA (Fig. 5). After 24 and 72 h of treatment, the amount of released LDH in cells cultured with nega-

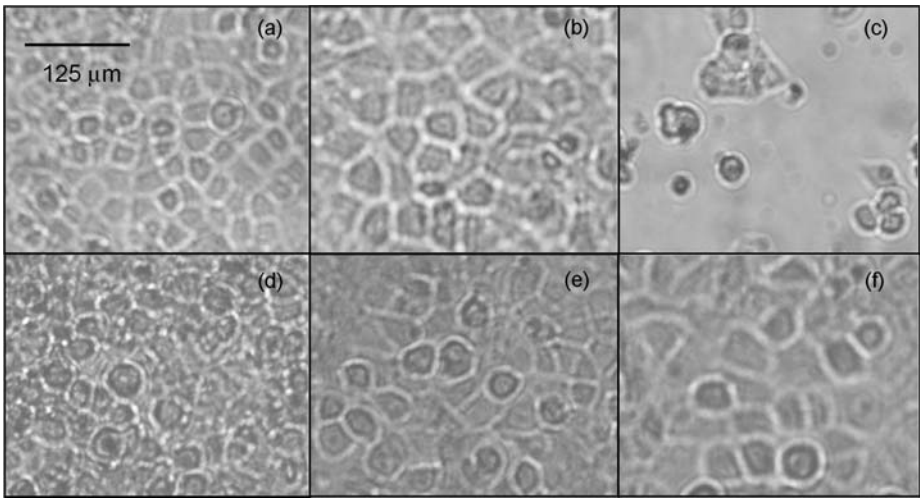

Fig. 4. Effect of (a) control, (b) negative control (high-density polyethylene), (c) positive control (PVC-containing organotin stabilizer), (d) fluor-hydroxyapatite, (e) fluorapatite and (f) hydroxyapatite on morphology of NIH-3T3 cells after 72 h treatment. Magnification: 160×.

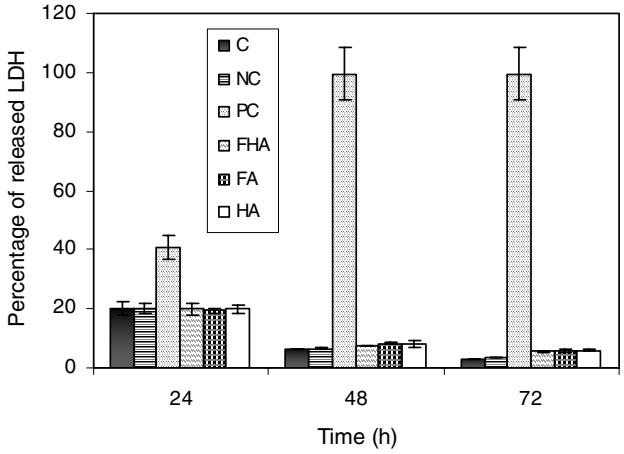

Fig. 5. The percentage of released LDH in cells influenced by control (C), negative control (NC, high-density polyethylene), positive control (PC, PVC-containing organotin stabilizer), fluor-hydroxyapatite (FHA), fluorapatite (FA) and hydroxyapatite (HA) after 24, 48 and 72 h treatment. Data represent mean values ± S.D. from three independent experiments (in each experiment three separate parallels were used for each type of biomaterial).

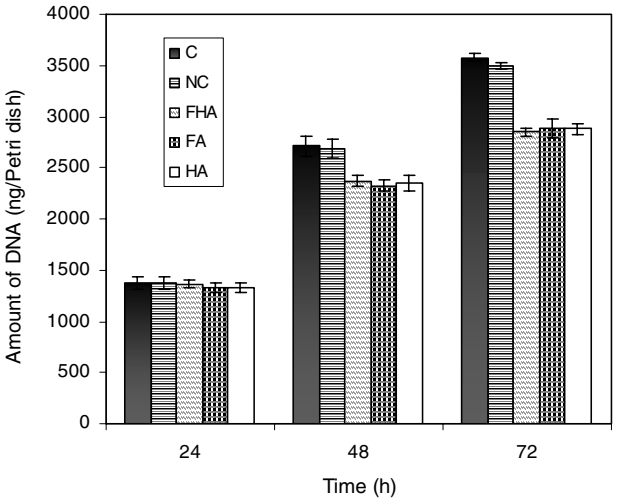

Fig. 6. The amount of DNA in cells after 24, 48 and 72 h treatment by control (C), negative control (NC, high-density polyethylene), fluor-hydroxyapatite (FHA), fluorapatite (FA) and hydroxyapatite (HA). Data represent mean values ± S.D. from three independent experiments (for each type of biomaterial three separate Petri dishes were used in each experiment).

tive control and biomaterials was not markedly changed (9.85–0.82%) in comparison with the amount of released LDH in non-affected cells. After 48 h of treatment, percentage of released LDH was in the range 16.3–16.6%. These observations correlated with the cell proliferation (Fig. 2) and DNA content (Fig. 6).

Direct effect of biomaterials on the content of cell proteins and DNA in NIH-3T3 cells

To study the metabolic activity of control, negative control and biomaterials-treated cells, at the same time we assessed the content of cell DNA (Fig. 6) and proteins (Fig. 7) together with determination of number of NIH-3T3 cells in the presence or in the absence of negative control, FHA, HA and FA. As can be seen from Figure 7, the cell protein content proportionally increased with culture time in affected cells in comparison with control. Negative control caused maximum 0.28% inhibition of the cell protein content during the whole experiment. After 72 h of treatment all tested biomaterials caused about 10% inhibition. Similarly, DNA content (Fig. 6) in control and affected cells increased with time of incubation with biomaterials, while in comparison with control about 20% inhibition was found in all treated cells after 72 h of treatment with all biomaterials. Negative control demonstrated maximum 0.49% inhibition of DNA content after 72 h of treatment. These observations correlated with the cell proliferation (Fig. 2).

Linear regression analysis of biomaterials treatment on relative growth of NIH-3T3 cells

For the mutual comparison of cytotoxicity of biomaterials FHA, FA and HA against control measurements (untreated cells), four cytotoxic methods were em-

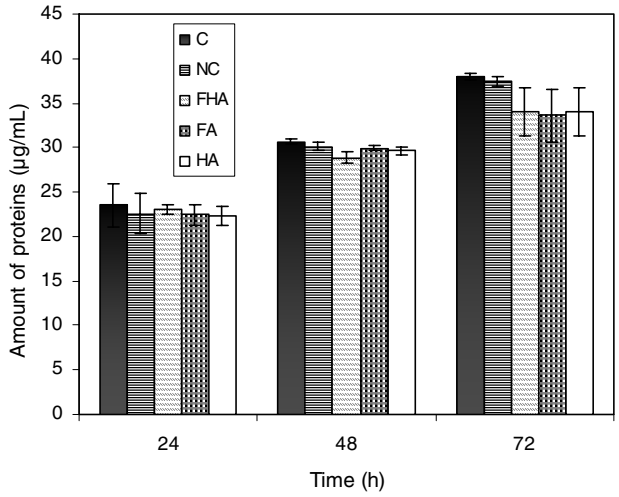

Fig. 7. The cell protein content after 24, 48 and 72 h treatment by control (C), negative control (NC, high-density polyethylene) and tested biomaterials fluor-hydroxyapatite (FHA), fluorapatite (FA) and hydroxyapatite (HA). Data represent mean values ± S.D. from three independent experiments (for each type of biomaterial three separate Petri dishes were used in each experiment).

ployed: the cell number, released LDH, protein content and DNA content (Fig. 8). Based on regression analysis, the parameters of regression equations, regression coefficients (R^2) and coefficients of variation (%CV) were calculated. As can be seen from Figure 8, all tested biomaterials showed certain cytotoxicity measured by used methods. From the course of regression lines (Fig. 8) it resulted that linear dependence for number of cells (A), DNA content (C) and protein content (D) are statistically significant while regression coefficents R^2 was in the range 0.73–0.99 and relative error of measurement CV was in the range 0.11–9.07%. The method of measurement of released LDH in all tested biomaterials was not statistically significant in time-dependence.

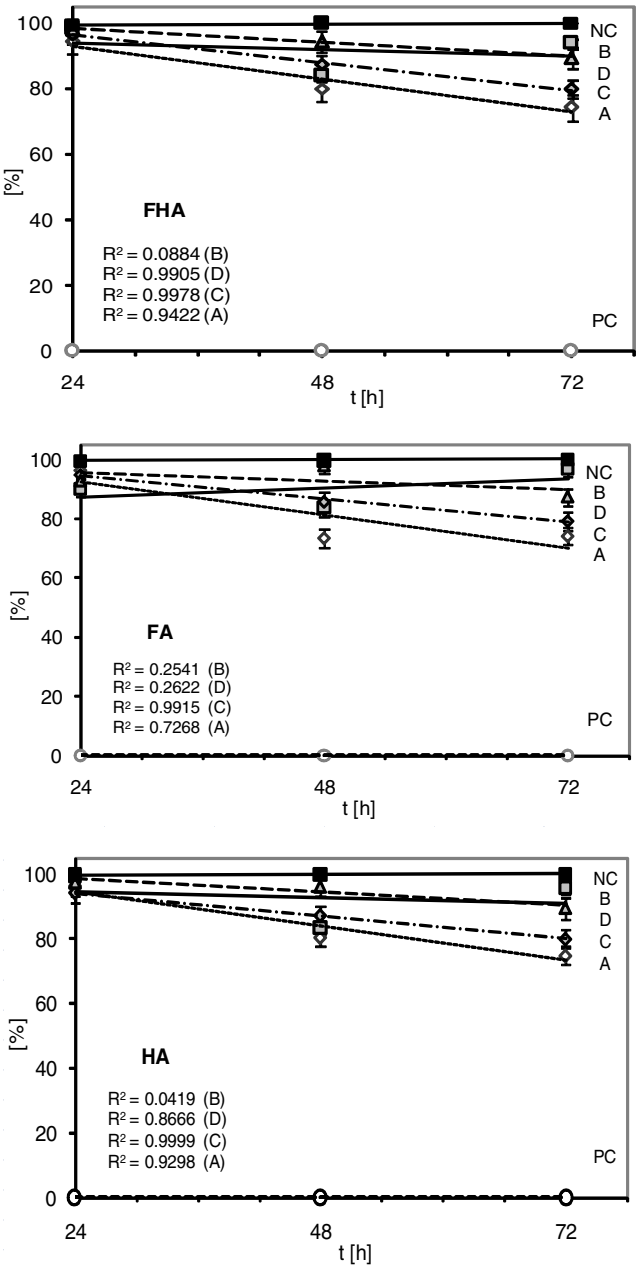

Fig. 8. Linear regression analysis of growth of treated cells related to the control. Growth was evaluated with four parameters: number of cells (A), released LDH (B), DNA content (C) and protein content (D). NC, negative control; PC, positive control.

Discussion

The number of polymers and other materials used in biomedical applications has rapidly increased in the past two decades. Biomaterials (ceramics and polymers) may have low, medium or high potential risk to human safety, depending on the type and extent of patient contact. Safety assessments of medical biomaterial are guided by the toxicological guidelines recommended in the International Organization for Standardization (ISO 10993-1/EN 30993-1). The subject of ISO 10993-1 provides a standardised battery of biological safety (biocompatibility) tests. Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application. Cytotoxicity testing represents the initial phase in testing biocompatibility of potential biomaterials and medical devices.

Generally, the ability of chemicals to possess toxic potential may be classified in several ways. One of them is the classification of primary toxic mechanisms based on different levels of organization in the human body which could be affected by chemical. According to that basal cytotoxicity, organ-specific cytotoxicity and organizational cytotoxicity are considered. Cellular basal cytotoxicity can be demonstrated as a result of chemical attack upon cell membrane integrity, mitochondrial activity, or protein or DNA synthesis, since these are examples of fundamental metabolic function which are common to all cells. Most cell lines of mesenchymal origin, for instance fibroblasts, without specialized persistent metabolic functions demonstrate reactions to toxic agents based on basal cell functions (Barile 1994). The L929 clone of mouse fibroblasts is commonly used to assess the cytotoxicity of an experimental material for

medical applications (ISO 10993-5; Rice et al. 1978; Vallittu & Ekstrand 1999; Liskorish et al. 2004; Nablo & Schoenfish 2005; Ignjatovič et al. 2006). Mouse L929 and Balb/c fibroblasts are appropriate cell models for the study of cellular basal cytotoxicity because of their easiness to maintain and good correlation with animal tests (INVITTOX protocols no.38, 46).

In the present work, the embryonal mouse fibroblast cell line NIH-3T3 was used for comparative study of basal cytotoxicity of fluoridated biomaterials FHA and FA discs. The appropriateness of the use of these cells and their sensitivity for tested biomaterials were evaluated on the basis of five cytotoxic end points: cell proliferation, cell morphology, LDH released, protein and DNA cell contents. The basal cytotoxicity of FHA, FA and HA discs was measured by direct contact method. After 24, 48 and 72 h, the cell growth was evaluated by direct counting of non-affected cells and cells treated by biomaterials. Simultaneously with the assessment of cell numbers the metabolic activity of cells, namely by the assessment of cell proteins and DNA content, was monitored. Viability of cells was evaluated on the basis of the released LDH. The growth rates of cell proliferation using primary model according to Baranyi et al. (1993) were calculated. Furthermore, linear regression analysis was done for relative growth of NIH-3T3 cells measured by four cytotoxic methods.

The obtained results showed that positive control (PVC-containing organotin stabilizer) induced strong cytotoxic effect which caused total lysis of cell population. On the other hand, negative control (high-density polyethylene) did not induce cytotoxicity; treated cells grew as control, untreated NIH-3T3 cells (Fig. 2). Tested biomaterials demonstrated different concentration- and time-dependent cytotoxic effects. After 24 h of treatment, the inhibition of cell proliferation at all biomaterials was found less than 10%, while after 72 h the inhibition of cell proliferation was about 25%. The growth rate of cells affected by biomaterials was about 20% lower than the growth rate of control, untreated NIH-3T3 cells.

Cytotoxic effects of FHA, FA and HA evaluated on the basis of direct counting of cell number, released LDH, cell protein content and DNA content increased with the time of treatment. After 24 h, biomaterials demonstrated slight (less than 10%) cytotoxic effect in the range 3.66–5.49% for cell number, 0.82–9.85% for released LDH, 2.1–7.39% for protein content and 4.53–6.0% for DNA content. On the other hand, after long-term influence, 48 h and 72 h, of biomaterials all tested biomaterials induced, respectively, 19.52–26.62% and 25.15–25.91% inhibition of cell proliferation (Fig. 2), 16.3–16.6% and 3.1–6.31% released of LDH (Fig. 5), 2.14–5.66% and 10.67–12.49% inhibition of cell protein content (Fig. 7) and 12.57–14.28% and 20.04–20.88% inhibition of cell DNA content (Fig. 6) in comparison to control. Linear regression analysis (Fig. 8) showed that all used cytotoxic methods showed nearly the same cytotoxicity of biomaterials; deviation of measurements was less than 10% for number cells, protein content, DNA content and 12.4% for released LDH, where linear course of curves was not so statistically significant.

Based on the obtained results of the present study it can be concluded that FHA composite, FA and HA demonstrated similar basal cytotoxicity increasing with the time of treatment in NIH-3T3 cell line. From the growth rate it resulted that NIH-3T3 cells affected by tested biomaterials divided about 20% slowlier than the control, untreated cells. After 72 h of biomaterials treatment, about 25% inhibition of cell number, unchanged morphology of dividing cells, about 5% increase of released LDH, about 10% inhibition of cell protein content and about 20% inhibition of DNA content were found. Linear regression analysis showed that deviations in measurements of cytotoxicity by four methods were less than 10% for cell number, protein content and DNA content methods and 12.4% for released LDH method.

The probable cause of not finding very different cytotoxicity of FHA, FA and HA was that from the surface of FHA and FA discs only the amount of fluoride ion was eluted by direct contact assay which with respect to pure HA did not express the increase in their basal cytotoxicity.

Different cytotoxicity of FHA, FA and FA was found when studying the cytotoxicity of their 5 day's eluates on mouse fibroblast NIH-3T3 cells and leukemia L1210 cells. Eluate assay on NIH-3T3 cells (Jantová S., Letašiová S., Theiszová M. & Palou M., unpublished results) showed that any of tested biomaterial 5 day's eluates did not cause the total inhibition of cell division. Biomaterials induced different antiproliferative effects increasing in the order HA<FHA<FA which were time- and concentration-dependent. None of the tested biomaterials induced necrotic/apoptotic death of NIH-3T3 cells. Eluate assay on leukemia L1210 cells (Theiszová M., Jantová S., Letašiová S. & Palou M., unpublished results) showed that biomaterial eluates induced concentration- and time-dependent inhibition of cell growth. Cytotoxicity of eluates increased in order FA<HA<FHA. FHA and FA induced programmed cell death through mitochondrial/caspase-9/caspase-3-dependent pathway.

The significance of fluoride ion in cytotoxicity of FA composites was demonstrated by many authors, too. Kim et al. (2004) reported that the cell proliferation rate of the osteoblastic MG63 cells on the FHA coating layer decreased slightly with increasing F-incorporation. Lee et al. (2005) also demonstrated that the osteoblast-like cells on the fluoridated coatings showed a lower proliferation level compared to those on the pure HA coating. Qu & Wei (2006) have used FHA discs with various fluoride contents (0–0.567 mol F^-/mol) to investigate their effects on osteoblastic cell behaviour. Osteosarcoma cells were cultured on FHA discs for different time periods and the cell proliferation, morphology and differentiation were examined. The authors found out that the cell attachment and proliferation were affected more on the fluoride-containing FHA discs than on pure HA.

These differences in cytotoxicity of fluoridated biomaterials in comparison to HA were probably caused by the presence and elution of fluoride from the structure of FHA and FA. It is known that fluoride is the substance with significant biological activity. Fluoride stimulates bone formation and is capable to increase substantially cancerous bone mass. Therefore it is used for osteoporosis treatment (Astern et al. 1998). Furthermore, fluoride has been widely used in dentistry as caries prophylactic agents (Ribeiro et al. 2004). NaF was the first and still most recommended fluoride compound for fluoridation of drinking water (Ribeiro et al. 2006). Fluoride has shown considerable variation in respect of cytotoxicity against different cultured cells (Hongslo et al. 2004; Oguro et al. 1983). Other manifestation of fluoride biological activity includes fluoride toxic effects – skeletal fluorosis and damage to kidney liver, parathyzoid glands and brain (Wang et al. 2004; Shan et al. 2004).

Based on the obtained results it can be concluded that FHA composite, FA and HA demonstrated in embryonal fibroblast cell line similar basal cytotoxicity increasing with the time of treatment. On the other hand, from the growth rates it resulted that NIH-3T3 cells affected by tested biomaterials divided about 20% slowlier than the control, untreated cells. Using linear regression analysis it was found that deviations in measurements of cytotoxicity by four methods were less than 10% for cell number, protein content and DNA content methods and 12.4% for released LDH method. On the basis of good correlation of the cytotoxicity of biomaterials obtained from all end points it could be concluded that fibroblast NIH-3T3 cell line was appropriate for measuring the basal cytoxicity of tested biomaterials.

Acknowledgements

This work was supported by Science and Technology Assistance Agency under the contract No. APVT 20-015904. We thank reviewers for valuable comments.

References

Astern P., Darroudi F., Natarajan A.T., Terpstra I.J. & Duursma S.A. 1998. Cytogenetic effects on lymphocytes in osteoporotic patients on long-term fluoride therapy. Pharm. World Sci. **20:** 214–218.

Baranyi J., Roberts T.A. & McClure P. 1993. A non-autonomous differential equation to model bacterial growth. Int. J. Food Microbiol. **10:** 43–59.

Barile F.A. 1994. Introduction to *in vitro* Cytotoxicology. Mechanism and Methods. CRC Press, Boca Raton, Florida, USA, 212 pp.

Bergmeier H.U. 1970. Methoden der enzymatischen Analyse, 2nd Ed. Akademie Verlag, Berlin.

Farley J.R., Wergedal J.E. & Baylink D.L. 1983. Fluoride directly stimulates proliferation and alkaline phosphatase activity of bone-forming cells. Science **222:** 330–332.

Gineste L., Gineste M., Ranz X., Ellefterion A., Guilhem A., Rouquet N. & Frayssinet P. 1999. Degradation of hydroxylapatite, fluorapatite, and fluorhydroxyapatite coatings of dental implants in dogs. J. Biomed. Mater. Res. **48:** 224–234.

Hongslo J.K., Holland R.I. & Jonsen J. 1974. Effect of sodium fluoride on LS cells. J. Dent. Res. **53:** 410–413.

Ignjatovič N., Ninkov P., Kojič V., Bokurov M., Srdič V., Krnojelac D., Selakovič S. & Uskokovič D. 2006. Cytotoxicity and fibroblast properties during *in vitro* test of biphasic calcium phosphate/poly-dl-lactide-co-glycolide biocomposites and different phosphate materials. Microsc. Res. Tech. **69:** 976–982.

Jarcho M., Kay J.F., Gumaer K.I., Doremus R.H. & Drobeck H.P. 1977. Tissue, cellular and subcellular events at a bone-ceramic hydroxylapatite interface. J. Bioeng. **1:** 79–92.

Karen J.L.B., Porter S. & Kellam J.F. 2000. Biomaterial developments for bone tissue engineering. Biomaterials **21:** 2347–2359.

Kuzielová E., Theiszová M. & Palou T.M. 2006. Thermal stability of hydroxyfluorapatite solid state solutions during sintering, cytotoxicity of fluorapatite, pp. 99–102. In: Book of Abstracts: 28. Mezinárodní slovenský a český kalorimetrický seminář, Univerzita Pardubice, Pardubice, ISBN 80-7194-859-4.

Labarca C. & Paigen K. 1980. A simple, rapid and sensitive DNA assay procedure. Anal. Biochem. **102:** 344–352.

Lang H. & Mertens T.H. 1990. The use of cultures of human osteoblast-like cells as an *in vitro* test system for dental materials. J. Oral Maxillofac. Surg. **48:** 606–611.

LeGeros R.Z. 1993. Biodegradation and bioresorption of calcium phosphate ceramics. Clin. Mater. **14:** 65–88.

LeGeros R.Z., Lin S., Rohanizadeh R., Mijares D. & LeGeros J.P. 2003. Biphasic calcium phosphate bioceramics: preparation, properties and applications. J. Mater. Sci. Mater. Med. **4:** 201–209.

Leroy N. & Bres E. 2001. Structure and substitutions in fluorapatite. Eur. Cell. Mater. **2:** 36–48.

Lickorish D., Ramshaw J.A., Werkmeister J.A., Glattauer V. & Hewlett C.R. 2004. Collagen-hydroxyapatite composite prepared by biomimetic process. J. Biomed. Mater. Res. **68A:** 19–27.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193:** 265–275.

Marugan R., Kumar T.S.S. & Rao K.P. 2002. Fluorinated bovine hydroxyapatite, preparation and characterization. Mater. Lett. **57:** 429–433.

Mobasherpour I., Soulati Heshajin M., Kazemzadeh A. & Zakeri M. 2007. Synthesis of nanocrystalline hydroxyapatite by using precipitation method. J. Alloys Comp. **430:** 330–333.

Nablo B. & Schoenfisch M.H. 2005. *In vitro* cytotoxicity of nitric oxide-releasing sol-gel derived materials. Biomaterials **26:** 4405–4415.

Ning C. & Dai K., 2003. Research development of hydroxyapatite-based composites used as hard tissue replacement. Sheng Wu Yi Xue Gong Cheng Xua Za Zhi **20:** 550–554.

Oguro A., Koizumi N. & Horii K. 1983. Effect of fluoride ion on proliferation of Vero cell line cells: growth acceleration by sodium fluoride. J. Dent. Health **31:** 453–460.

Pereira M.M., Jones J.R. & Hench L.L. 2005. Bioactive glass and hybrid scaffolds prepared by sol-gel method for bone tissue engineering. Adv. Appl. Ceramics **104:** 35–42.

Qu H. & Wei M. 2006.The effect of fluoride contents in fluoridated hydroxyapatite on osteoblast behavior. Acta Biomater. **2:** 113–119.

Rejda B.V., Peelen J.G. & de Groot K. 1977. Tri-calcium phosphate as a bone substitute. J. Bioeng. **1:** 93–97.

Ribeiro D.A., Alves de Lima P.L., Marques M.E.A. & Salvadori D.M.F. 2006. Lack of DNA damage induced by fluoride on mouse lymphoma and human fibroblast cells by single cell gel (Comet) assay. Braz. Dent. J. **17:** 91–94.

Ribeiro D.A., Scolastici C., Marques M.E. & Salvadori D.M. 2004. Fluoride does not induce DNA breage in Chinese hamster ovary cells *in vitro*. Braz. Oral. Res. **18:** 192–196.

Rice R.M., Hegyeli A.F., Gourlay S.J., Wade C.W., Dillon J.G., Jaffe H. & Kulkarni R.K. 1978. Biocompatibility testing of polymers: *in vitro* studies with *in vivo* correlation. J. Biomed. Mater. Res. **12:** 43–54.

Shan K.R., Qi X.L., Long Y.G., Nordberg A. & Guan Z.Z. 2004. Decreased nicotinic receptors in PC12 cells and rat brains influenced by fluoride toxicity-a mechanism relating to a damage at the level in post-transcription of the receptor genes. Toxicology **200:** 169–177.

Tadashi K., Kim H.M. & Kawashita M. 2003. Novel bioactive materials with different mechanical properties. Biomaterials **24:** 2161–2175.

Vallittu P.K. & Ekstrand K. 1999. *In vitro* cytotoxicity of fibre-polymethyl methacrylate composite used in dentures. J. Oral. Rehabil. **28:** 666–671.

Wang A.G., Xia T., Chu Q.L., Zhang M., Liu F., Chen X.M. & Yang K.D. 2004. Effects of fluoride on lipid peroxidation, DNA damage and apoptosis in human embryo hepatocytes. Biomed. Environ. Sci. **17:** 217–222.

Xu W., Hu W., Li M. & Wen C. 2006. Sol-gel derived hydroxyapatite/titania biocoatings on titanium substrate. Mater. Lett. **60:** 1575–1578.

Received October 8, 2007
Accepted January 18, 2008