# Cytotoxicity of hydroxyapatite, fluorapatite and fluor-hydroxyapatite: a comparative *in vitro* study.

M. THEISZOVA[1], S. JANTOVA[1], S. LETASIOVA[1], M. PALOU[2], L. CIPAK[3,*]

*[1] Institute of Biochemistry, Nutrition and Health Protection, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinskeho 9, 812 37 Bratislava, Slovakia; [2] Institute of Inorganic Chemistry, Technology and Materials, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinskeho 9, 812 37 Bratislava, Slovakia; [3] Cancer Research Institute, Slovak Academy of Sciences, Vlarska 7, 833 91 Bratislava, Slovakia, exoncip@savba.sk*

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The purpose of this study was to evaluate the cytotoxicity of two formulations of hydroxyapatite (HA), namely fluorapatite (FA) and fluor-hydroxyapatite (FHA). HA is used as carrier material for antibiotics or anticancer drugs during treatment of bone metastasis. Negative control, represented by HA, was included for comparative purposes. Leukemia cells were used as a model cell line, and the effect of eluates of tested biomaterials on cell proliferation/viability and mechanism of antiproliferative activity were assessed. Study design attempted to reveal the toxicity of tested biomaterials with an emphasis to decide if tested biomaterials have promise for further studies *in vivo*. Results showed that eluates of FA and FHA inhibit the growth of leukemia cells and induce programmed cell death through mitochondrial/caspase-9/caspase-3-dependent pathway. Due to these differences compare to HA, it is concluded that FA and FHA have promise for evaluation of their behaviour *in vivo*.

*Keywords: hydroxyapatite; fluorapatite; fluor-hydroxyapatite; cytotoxicity; apoptosis*

Hydroxyapatite (HA) has been extensively investigated as suitable material for repair of bone defects [1]. It has been shown to stimulate osteoconduction and is a material that can be integrated into bone without provoking an immune reaction. Additionally, its derivatives can be used to administer antibiotics [2] or anticancer drugs [3–6], with the aim of both repairing bone defects and inhibiting cancer cell growth. Previously, it has been shown that nanoscale HA crystallite could inhibit the growth of cancer cells (e.g. hepatoma, colon cancer and osteosarcoma) while having little effects on normal cells [7–10]. However, the low mechanical strength of HA crystallite does not alow to form a fixed shape in human body hindering its clinical applications.

Recently, fluorapatite (FA) and fluor-hydroxyapatite solid solutions (FHA) as alternative bioceramics in replacement of pure HA have been investigated [11]. FA is considered as biomedical material due to its structure similar to HA. In the case of 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 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 or tooth enamel. Additionally, the fluoride is uniformly distributed within the bone tissue and thin tooth enamel outer layer. Apart from bioactivity, these biomaterials show higher thermal resistance and better mechanical properties compare to pure HA bioceramics, thus seeming to be preferable materials for repair of bone defects and cancer treatment.

It is well known from cell culture studies, that the biomaterial particles reduce cell adhesion and viability significantly when compared to the cells that are not exposed to the particles. One possible explanation for decrease in cell adhesion and viability is that these particles are taken up by the cells as a result of endocytosis. Endocytosis of the particles results in disruption of the cell membrane and disorganized cell cytoskeleton, followed by apoptosis [12]. This contact/endocytosis-induced acute toxicity is masking the long-term chronic toxicity of biomaterial particles. Despite, the particles are considered as inert material, the chance of their biodegradation and possible elution of some chemicals in time still exists. This may represent significant risk to humans treated with biomaterials.

* Corresponding author

In the present study, the cytotoxic properties of eluates of prepared formulations of hydroxyapatite, namely fluorapatite and fluor-hydroxyapatite, were assayed with an emphasis to decide if these biomaterials have promise for further studies *in vivo*. To evaluate their toxicity, we monitored the effect of the eluates on cell proliferation/viability and elucidated the mechanism of their antiproliferative activities.

## Materials and methods

*Cells.* The murine leukemia L1210 cell line, obtained from the ATCC (Rockville, MD, USA), was cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (292.3 μg/ml), and 10% heat-inactivated fetal calf serum in an atmosphere of 5% $CO_2$ in humidified air at 37°C. The cell growth and viability were assessed by 0.4% trypan blue staining.

*Preparation of biomaterials.* Hydroxyapatite (HA) 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 agent for pH adjustment [13]. Equation illustrates chemical reaction leading to the precipitation of HA.

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

A suspension of $Ca(NO_3)_2 \cdot 4H_2O$ powder was diluted in deionized 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 pH 10. The final solution was stirred at room temperature for 3 h. Then, the precipitate formed was filtered, washed with deionized water several times (neutral pH), and finally dried under IR lamp for 24 h. After drying, the sample was powdered and treated at 900°C for 1 h. The obtained product was controlled by powder X-ray diffraction (XRD). HA has been identified as Hap JCPDS 9-438.

Fluorapatite (FA) was obtained using $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ according to the following equation:

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

The solid solutions of fluor-hydroxypaptite (FHA), $Ca_{10}(PO_4)_6(OH)F$, were prepared by precipitation method according to the following equation:

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

HA, FA and FHA (porous size was less than 125 μm) were used for preparation of 5-days' concentrated eluates. Culture medium supplemented with penicillin and streptomycin was used. Biomaterial powders were sterilized for 30 min at 130°C, then the cultivation medium was added and samples were shaken on reciprocal shaker for 5 days at 37°C. After 5 day elution, the concentrated sample was centrifuged (10 min, 1100 g), the culture medium was aspirated by syringe and filtered (∅ 0.22 μm). This procedure led to preparation of 100 mg/ml of biomaterial eluates (HA, FA and FHA). Eluates were stored at -20°C.

*Antiproliferative effect.* L1210 cells were placed at Petri dishes (∅ 60 mm) at a density of 0.8 x $10^5$ cells/ml. After 24 h of incubation, the cells were exposed to 10, 25, 50, 75 and 100 mg/ml of biomaterial eluates. After 24-72 h treatment, the cells were examined under microscope. The number of cells and their viability were assessed by direct counting of cells using trypan blue staining.

*Flow cytometry.* Untreated and cells treated with 5, 10, 25, 50, 75, 100 mg/ml of biomaterial eluates were harvested, washed twice with PBS and exposed to 0.1% Triton X-100 (Sigma Chemical, St Louis, MO, USA) in PBS supplemented with RNAse (50 μg/ml, Sigma) for 25 min at 37°C. Afterwards, DNA was stained with propidium iodide (PI) (50 μg/ml, Sigma) for 15 min at 4°C. Samples were analyzed by Coulter Epics XL flow cytometer (Beckman Coulter Company, Miami, Florida, USA) with the use of DNA Cell Cycle Analysis Software (Phoenix Flow Systems – MultiCycle AV for Windows). A minimum of 10000 cells per sample were analyzed at a flow rate of 200 cells/s.

*Caspase-3 activity assay.* Cells were treated with biomaterial eluates for 72 h. Cell lysates were prepared and caspase-3 activity was measured according to the manufacturer's protocol (CaspACETM Assay System, Promega Corporation, USA). Briefly, 28 μg of total protein (10 μl) was added to the reaction mixtures containing colorimetric substrate peptides specific for caspase-3 (Ac-DEVD-pNA) at 37°C for 2, 4, 6 h. Absorbance at 405 nm was determined using microplate reader (Humareader, Wiesbaden, SRN). Protein concentration was determined by Lowry et al. [14].

*Caspase-8 and caspase-9 activity assays.* Cells were treated with biomaterial eluates for 72 h. Caspase-8 and caspase-9 activities were measured according to the manufacturer's protocol (CaspACE™ Assay System, Promega Corporation, USA). Briefly, 100 μl of Caspase – Glo™ 8 Reagent (Ac-LETD-pNA) and 100 μl of Caspase – Glo™ 9 Reagent (Ac-LEHD-pNA), respectively, were added to the test tube with 100 μl of cell suspension containing 50000 cells, mixed and the luminiscent signal was measured immediately after mixture and in 10, 30, 60 min.

*Statistics.* Results are shown as the arithmetic means ± s.d. of the mean of three separate experiments (for each concentration of biomaterial three separate Petri dishes were used). Statistical analysis was performed by the Kruskal-Wallis one-way ANOVA test for nonparametric measurements ($H > 3.86$, $P < 0.05$ was considered statistically significant). The antiproliferative effect of biomaterial eluates was analyzed by Friedman's nonparametric test ($P < 0.0037$ was considered statistically significant).

## Results

*Antiproliferative effects of biomaterial eluates.* Viability of cells exposed to biomaterial particles decreased by effects of

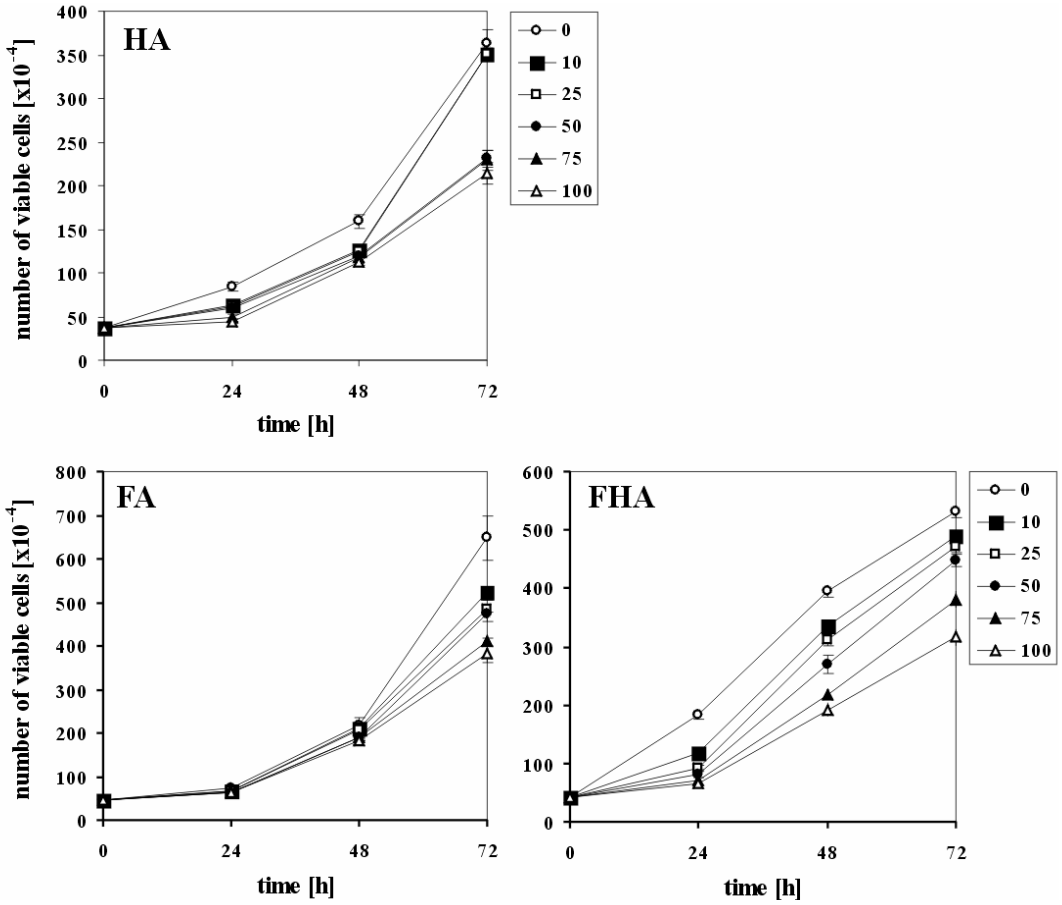


**Figure 1. Effect of hydroxyapatite (HA), fluorapatite (FA) and fluor-hydroxyapatite (FHA) (10 – 100 mg/ml) was evaluated on growth of L1210 leukemia cells. Data represent mean values ± s.d. of three independent experiments.**

particles on cell adhesion or as a result of particle endocytosis [12]. Out of these factors, the chemicals eluted from particles may represent significant factor contributing to their toxic effects. Our previous study demonstrated that HA, FA and FHA particles did not induce significant toxicity to adherent cells [15]. To test if components of particles are eluted and if they are toxic, the concentrated 5-days' eluates of HA, FA and FHA were prepared and tested for their toxicity on L1210 leukemia cells. This cell line was chosen due to advantages, e.g. higher sensitivity to toxic agents and quick stress response compare to adherent cells. As presented in Fig. 1, prepared biomaterial eluates induced concentration- and time-dependent inhibition of cell growth. Toxicity of eluates increased in the order FA < HA < FHA. The undilluted eluates (100 mg/ml) inhibited the growth of cells after 72 h exposure to 82.2% for FHA, 80% for HA and 40% for FA, respectively. Viability staining and flow cytometry analysis revealed that eluates did not induce significant changes in cell cycle profile (data not shown). Instead, the cell death (detected as sub-$G_0$ cell population, Fig. 2) was responsible for decreased number of cells treated with eluates of FA and FHA.

*Mechanism of antiproliferative activity of biomaterial eluates.* In the next set of experiments, we tried to elucidate the mechanism of cytotoxicity induced by eluates. To investigate whether sub-$G_0$ cell population contains the cells dying by apoptosis, treated cells were subjected to agarose gel electrophoresis and Hoechst 33258/PI staining. Apoptotic DNA fragmentation and apoptotic bodies were detected in cells treated with 50 – 100 mg/ml of FA and 10 – 100 mg/ml of FHA. In HA-treated cells, no features of DNA fragmentation or apoptotic bodies were detected (data not shown). To confirm the apoptosis in cells treated with FA and FHA, the cells were investigated for activation of caspases. As presented in Fig. 3A, the caspase-3 activity induced by FA was comparable with the caspase-3 activity induced by 1.8 mg/ml of cisplatin. On the other hand, the 75 – 100 mg/ml of FHA caused higher caspase-3 activity compare to cisplatin. Furthermore, the increase in caspase-9 activity was detected in cells treated with 50 – 100 mg/ml of FA and FHA (Fig. 3B). Activation of caspase-8 was not detected (data not shown).

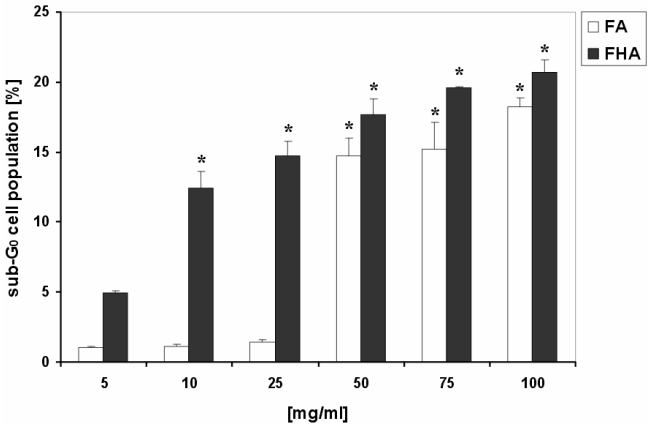


**Figure 2. Induction of apoptosis by fluorapatite (FA) and fluor-hydroxyapatite (FHA) after 72 h exposure in L1210 leukemia cells monitored as sub-$G_0$ cell population. Data represent mean values ± s.d. of three independent experiments.**

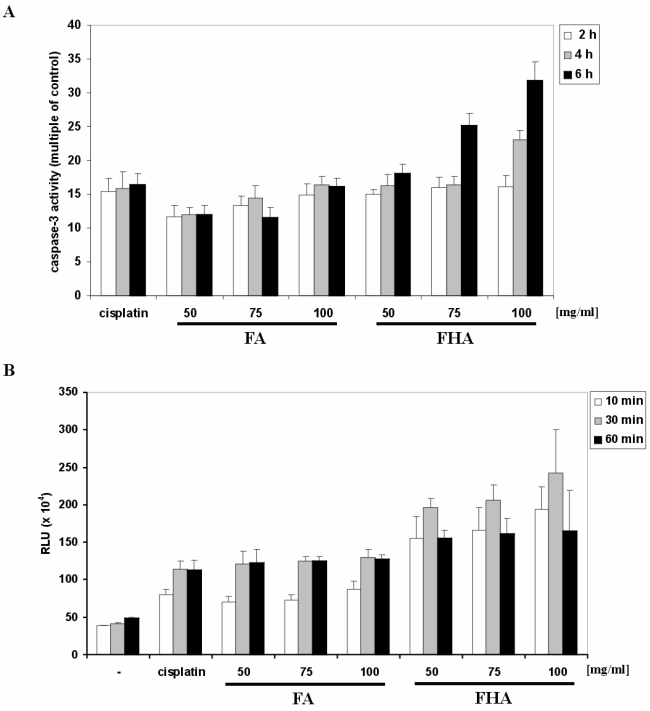


**Figure 3. Activation of caspase-3** (A) **and caspase-9** (B) **in L1210 leukemia cells treated with fluorapatite (FA) and fluor-hydroxyapatite (FHA) for 72 h. Cisplatin = 1.8 mg/ml, RLU = relative luminiscent signal. Data represent mean values ± s.d. of three independent experiments.**

## Discussion

Initially, hydroxyapatite (HA), fluorapatite (FA) and various solid-solutions of fluor-hydroxyapatite (FHA) have been prepared by precipitation method [13]. Using adherent cells as model cell line to investigate the toxic effects of these biomaterials, no significant cytotoxicity was observed. Despite their low cytotoxicity, the highest concentrations of biomaterials induced significant damage of DNA [15].

To discriminate between cytotoxicity due to decreased adherence of cells or particles endocytosis and cytotoxicity of eluted components of biomaterials (during the incubation *in vitro*), we decided to prepare eluates of biomaterials and evaluate their cytotoxicity towards L1210 leukemia cells. Our study demonstrated that eluates of biomaterials induce concentration- and time-dependent inhibition of cell growth (Fig. 1). This finding demonstrates that chemicals eluted from prepared biomaterials are able to inhibit the growth of leukemia cells, despite of weak effect of particles on adherent cells [15]. Recently, the growth inhibition effect of nanoscale HA towards cancer cell lines was reported [7–11]. Comparison of the inhibition growth effects of HA with different porous size on U2OS (from nanoscale to micrometer), showed greater activity of nanoscale HA. The inhibition rate of nanoscale HA increased with time and reached 73% after 72 h. On the other hand, the inhibition rate of micron HA decreased with time and reached 21% after 72 h. These findings are consistent with our results, and clearly demonstrate that cytotoxicity of nanoscale particles of HA is due to endocytosis, in contrast to micrometer HA.

The inhibition effects of FA and FHA on growth of cancer cell lines have not been demonstrated so far. In our study, eluate of FHA was less effective than eluate of HA, and eluate of FA was as effective as eluate of HA on leukemia L1210 cells. The rationale behind these results could be attributed to differences in biodegradation of materials tested. It is worth to mention, that elution of chemicals from biomaterials is time-dependent and toxicity of such biomaterials can be easily overlooked, if adherent cells or short time of exposure is applied. Therefore, the approach of simultaneous testing of biomaterial eluates and biomaterial particles on adherent and suspension cells is highly desirable.

Previous studies demonstrated that nanoscale hydroxyapatite and some of hydroxyapatite compounds may induce apoptosis of cancer cell lines [7–11]. On the basis of our observation, that cells treated with eluates of FA or FHA contain sub-$G_0$ cell population (Fig. 2), in the next experiments we elucidated the mechanism of their antiproliferative activities. Our results clearly showed that eluates of FA and FHA, in contrast to eluate of HA, induce apoptosis of leukemia cells, detected as apoptotic DNA fragmentation/apoptotic bodies formations and activations of caspase-3 and caspase-9 (Fig. 3). Since eluates of FA and FHA are cytotoxic and are inducing programmed cell death, further studies of their behaviour *in vivo* are necessary.

Taken together, our results indicate that prepared eluates of FA and FHA have cytotoxicity comparable to HA, however in contrast to HA they induce apoptosis of leukemia cells. This can be benefit in addition to their higher

thermal resistance and better mechanical properties, for their use as materials for repair of bone defects and cancer treatment.

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## References

[1] SUN JS, LIN FH, HUNG TY et al. The influence of hydroxyapatite particles on osteoclast cell activities. J Biomed Mater Res 1999; 1: 27–34.

[2] ITOKAZU M, AOKI T, NONOMURA H et al. Antibiotic-loaded porous hydroxyapatite blocks for the treatment of osteomyelitis and postoperative infection. A preliminary report. Bull Hosp Jt Dis 1998; 57: 125–129.

[3] XIA Q, NIE H, CHEN D et al. The effect of locally implanted complexes of adriamycin-hydroxyapatite complexes on tumor-study of a new alternative treatment for hepatic cancer. J Tongji Med Univ 1999; 19: 219–222.

[4] UCHIDA A, SHINTO Y, ARAKI N, ONO K. Slow release of anticancer drugs from porous calcium hydroxyapatite ceramic. J Orthop Res 1992; 3: 440–445.

[5] YAMAMURA K, IWATA H, OSADO T et al. Anticancer effects of adriamycin-loaded hydroxyapatite implants determined in a swarm rat chondrosarcoma model. Jpn J Pharmacol 1994; 3: 289–291.

[6] TAHAR Y, ISHII Y. Apatite cement containing cis-diamminedichloroplatinum implanted in rabbit femur for sustained release of the anticancer drug and bone formation. J Orthop Sci 2001; 6: 556–565.

[7] LIU ZS, TANG SL, AI ZL. Effects of hydroxyapatite nanoparticles on proliferation and apoptosis of human hepatoma BEL-7402 cells. World J Gastroenterol 2003; 9: 1968–1971.

[8] INAYAT-HUSSAIN SH, RAJAB NF, ROSLIE H et al. Cell death induced by hydroxyapatite on L929 fibroblast cells. Med J Malaysia 2004; 59B: 176–177.

[9] FU Q, ZHOU N, HUANG W et al. Preparation and characterization of novel bioactive bone cement: Glass based nanoscale hydroxyapatite bone cement. J Mater Sci 2004; 15: 1333–1338.

[10] CHEN X, DENG CH, TANG S, ZHANG M. Mitochondria-dependent apoptosis induced by nanoscale hydroxyapatite in human gastric cancer SGC-7901 cells. Biol Pharm Bull. 2007; 1: 128–132.

[11] GROSS KA, RODRIGUEZ-LORENZO LM. Sintered hydroxyfluorapatites. Part I: Sintering ability of precipitated solid solution powders, Biomaterials 2004; 25: 1375–1384.

[12] BERRY CC, WELLS S, CHARLES S, CURTIS AS. Dextran and albumin derivatised iron oxide nanoparticles: influence on fibroblasts in vitro. Biomaterials 2003; 24: 4551–4557.

[13] KUZIELOVA E, PALOU MT. Microstructure development of fluor-hydroxyapatite solid solution during the sintering. VI Proceeding of conference on preparation of ceramic materials 2005; 16–17.

[14] LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 143: 265–267.

[15] THEISZOVA M, JANTOVA S, DRAGUNOVA J et al. Comparison the cytotoxicity of hydroxyapatite measured by direct cell counting and MTT test in murine fibroblast NIH-3T3 cells. Biomed Pap Med 2005; 2: 393–396.