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Cytotoxicity of a titanium alloy coated with hydroxyapatite by plasma jet deposition

Citotoksičnost legure titana obložene hidroksiapatitom pomoću mlaza plazme

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Abstract

Background/Aim. The deposition of hydroxyapatite (HAP) on the surface of titanium (Ti) alloys enhances bioactivity and osseointegration of the alloys which are widely used as implant materials in dentistry and orthopaedic surgery. However, the stability of HAP and subsequent biocompatibility of such alloys depends on the coating technique. The aim of this work was to test the cytotoxicity of a Ti alloy (Ti6Al4V), coated with HAP by a new plasma deposition method. **Methods.** The Ti6Al4V samples prepared as discs, 10 mm in diameter and 2 mm in thickness, were coated with HAP (one or both sides of the alloy) by an innovative atmospheric plasma jet method. The cytotoxicity of uncoated and HAP coated Ti6Al4V samples was evaluated by examining the morphological changes and viability of L929 fibroblasts in direct contact with the test materials. Adequate negative (polystyrene) and positive (nickel) control discs of the same size were used. The indirect cytotoxicity was determined by cultivating L929 cells with conditioning medium (CM), prepared as extract of the test samples incubated in the complete Roswell Park Memorial Institute (RPMI) 1640 medium for cell cultures. The cytotoxic effect was evaluated based on the degree of metabolic activity, necrosis, apoptosis and proliferation of L929 cells, using the appropriate assays. **Results.** Uncoated and one side HAP coated Ti6Al4V alloys were classified as non-cytotoxic according to the current ISO 10993-5 criteria, whereas two sides HAP coated Ti6Al4V alloy samples were slightly-moderate cytotoxic. The cytotoxicity manifested as the inhibition of metabolic activity and proliferation of L929 cells as well as the induction of their apoptosis and necrosis was significantly reduced by conditioning of HAP/Ti6Al4V alloys for 24 hours. The cytotoxic effect of HAP/Ti6Al4V CM only partly decreased in the presence of nifelate, a calcium (Ca) channel blocker, suggesting that Ca ions were not the only responsible cytotoxic agent. **Conclusion.** The original HAP coating procedure by atmospheric plasma spraying with high energy input enables the production of the stable adhesive coatings on Ti6Al4V alloys. Their cytotoxicity, which depends on the quantity of HAP coating layer, could be significantly reduced up to the non-cytotoxic level by prior conditioning of the alloys in culture medium. Such a procedure, which removes leachable toxic components, could be useful before implantation of HAP coated alloys *in vivo*.

Key words:
dental alloys; titanium; materials testing; hydroxyapatites.

Apstrakt

Uvod/Cilj. Oblaganje površine legura titana (Ti) hidroksiapatitom (HAP) poboljšava bioaktivnost i oseointegraciju Ti legura, koje se široko koriste kao implantacioni materijali u stomatologiji i ortopediji. Međutim, stabilnost HAP prevlake i biokompatibilnost takvih legura zavise od primenjene tehnike oblaganja. Cilj ovog rada je bio da se ispita citotoksičnost Ti6Al4V legure obložene sa HAP pomoću plazme korišćenjem originalne metode. **Metode.** Uzorci Ti6Al4V legure u obliku diska, prečnika 10 mm, debljine 2 mm su presvučeni sa HAP (jednostrano ili obostrano) mlazom atmosferske plazme. Citotoksičnost neobložene i HAP-om obloženih Ti6Al4V legura je ispitivana na osnovu morfoloških karakteristika i vijabilnosti L929 fibroblasta u direktnom kontaktu ćelija sa test materijalima. Odgovarajuća

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negativna kontrola (polistirenski diskovi) i pozitivna kontrola (diskovi od nikla) istih veličina kao i diskovi Ti6Al4V legura su takođe uključeni u eksperimente. Indirektna citotoksičnost je procenjivana nakon kultivisanja L929 ćelija sa kondicioniranim medijumom (CM), koji je predstavljao ekstrakt testiranih uzoraka inkubiranih u kompletnom Roswel Park Memorial Institute (RPMI) 1640 medijumu za ćelijske kulture. Citotoksični efekat CM je procenjivan na osnovu stepena metaboličke aktivnosti, nekroze, apoptoze i proliferacije L929 ćelija, korišćenjem adekvatnih testova. **Rezultati.** Neobložena Ti6Al4V legura i Ti6Al4V legura obložena jednostrano sa HAP su okarakterisane kao necitotoksične na osnovu ISO 10993-5 kriterijuma, dok je Ti6Al4V legura obložena sa HAP obostrano pokazivala blagu do umerenu citotoksičnost. Citotoksičnost, koja se manifestovala smanjenjem metaboličke aktivnosti i proliferacije L929 ćelija kao i indukcijom njihove apoptoze i nekroze, je bila značajno smanjena ako su uzorci HAP-om presvučenih legura kondicionirani u medijumu u toku 24 časa. Citotoksičnost CM pripremljenih od Ti6Al4V legura obloženih sa HAP je bila samo delimično smanjena u prisustvu nifelata, blokatora kalcijumovih (Ca) kanala, što ukazuje da Ca joni nisu jedini citotoksični faktor. **Zaključak.** Originalna metoda oblaganja Ti6Al4V legure sa HAP pomoću atmosferske plazme u obliku spreja visoke energije omogućava stabilnu adheziju prevlake. Citotoksičnost ovako obrađene legure, koja zavisi od količine nanetog HAP, se može znatno smanjiti do necitotoksičnog nivoa prethodnim kondicioniranjem u medijumu. Ova procedura, kojom se uklanjaju rastvorljive toksične komponente, može biti korisna pre *in vivo* implantacije legura obloženih sa HAP.

Ključne reči:
legure, stomatološke; titan; materijali, testiranje; hidroksiapatiti.

Introduction

Due to their good physical and mechanical properties and biocompatibility [1, 2], titanium (Ti) and its alloys are widely used as implant materials, predominantly in orthopaedic and dental implant surgery. However, their corrosion properties in acid or alkaline solutions and biological fluids are not satisfactory. Such biomaterials show high friction coefficient, poor wear properties and low abrasion resistance *in vivo* [3, 4]. These disadvantages can be overcome by modification of Ti or Ti alloy surface by different methods, which can lead to favourable bone regeneration and integrity between the bone tissue and implant surface. At the same time, these procedures can improve the clinical performance of implants, without affecting their original biocompatibility. Ideally, surface modifications should enhance osseointegration and fasten the healing phase after implantation. Biofunctionality of coating depends on the coating technique and factors that influence the process of osseointegration such as surface microroughness, coating thickness and nanotopography [5].

Deposition of hydroxyapatite (HAP) coatings on the surface of Ti alloys, due to its similarity to the biological apatite, enhances implant bioactivity and bonding with bone [3–5]. Besides, HAP coatings with corresponding roughness improve the primary stability of implants and can also induce osteogenic differentiation of human mesenchymal stem cells [6–8] and promotion of the ingrowth of new bone tissue [9]. The nanoscale features of HAP coatings increase the rate of the bone formation around the implant surface, diminishing particularly the friction in contact of the implants with natural bone and reducing significantly wearing of bone tissue in direct contact with implant [10]. Plasma-sprayed HAP coatings frequently show a large variation in coating thickness and density, insufficient coating-metal adhesion strength as well as changes in structural and chemical properties during the coating process, especially transformation of the crystalline structure of HAP to the amorphous phase [11, 12].

In our previous paper we described the microstructure and sintering mechanism of HAP coatings on pure Ti obtained by an innovative atmospheric plasma jet method with a high electric energy input [13]. The coating was stable because its adhesion was unusually high, around 60 MPa. Due to such a high adhesion, the coating was stable. On the other hand, the phase composition was appropriate because the crystalline phase of HAP predominates over the amorphous one. In this work, a similar procedure was applied for coating of the Ti6Al4V alloy. The principal goal of the study was to investigate cytotoxicity of uncoated, one side and two sides HAP coated TiV4Al6 test samples using L929 as a target cell line, recommended for the biocompatibility testing of medical devices [14], in different *in vitro* models.

Methods

HAP coatings of Ti6Al4V by plasma jet deposition

The rods of DC Ti6Al4V alloy (Bien-Air Medical Technologies, Switzerland) were cut into discs, diameter 10 mm and height 2 mm, by means of electro-erosion. The discs were firstly polished on one or both sides using fine aluminium oxide (Al_2O_3) powders with granulation of 5 –0.05 mm. The specimens were then immersed in 5 M NaOH aqueous solution for 24 h at 60°C after which they were removed from the solution and washed with distilled water. Afterwards, they were immersed in 1 M $Ca(NO_3)_2$ aqueous solution for 24 h at 60°C, rinsed with distilled water after removing from the solution, and dried at room temperature. Finally, the specimens were heated at 600°C for 4 h in the electrical furnace in an air atmosphere, and cooled to room temperature in the furnace.

Prepared titanium alloy (Ti6Al4V) specimens were further used as a substrate for plasma jet deposition of HAP. The plasma installation PJ-100 (Plasma Jet, Serbia) was used for the plasma spray process. The basic parameters of installation used for the coating deposition were: Plasma power 52.0 ± 1.5 kW, voltage 120 ± 2 V, current 430 ± 5 A, argon flow 38.5 ± 1.2 L/min, powder carrier gas (air) 8 L/min and powder feed rate 2.0 ± 0.1 g/s. The diameter of aperture of the anode nozzle was 8 mm, while the length of plasma jet was between 60 and 70 mm. The spraying process was controlled fully by a computer-driven device which enabled the nozzle to be moved at chosen speed and direction. Commercially available HAP powder (Captal® 90, Plasma Biotal Limited, UK), with an average grain size of 90 µm was used for the plasma deposition.

Test samples and conditioning

Test samples included: uncoated Ti6Al4V; one side and two sides HAP coated Ti6Al4V discs; polystyrene discs (negative control) and nickel (Ni) discs (positive control). All discs had the same dimensions, diameter of 10 mm and height of 2 mm. The polystyrene discs were prepared by cutting a polystyrene plate (Sarstedt, Germany). The nickel discs were prepared from a Ni road using the same procedure as used for cutting of the Ti6Al4V alloy. Before use in the cell culture experiments, the samples were sonicated in distilled water in an ultrasonic bath for 10 minutes, washed again with distilled water, sterilized in 70% alcohol for 30 minutes and transferred into the sterile Petri dishes to dry on ambient temperature.

The extracts of test samples in culture medium, named as conditioned medium (CM), were prepared by incubating the test samples in complete culture medium consisting of Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Munich, Germany) with addition of 10% foetal calf serum (Sigma), 2 mM of L-glutamine (Sigma) and antibiotics (penicillin, streptomycin and gentamicin) (all from Galenika, Belgrade), in an incubator with carbon dioxide (CO_2) at 37°C. The surface area of test samples/volume of complete medium was $1cm^2$/mL. Conditioning lasted either 24 hours or 7 days. A similar procedure was applied for the preparation of medium extract from already conditioned samples. After the incubation, CM were collected, centrifuged at 3000 rpm/ 10 min and then frozen at -20°C until use in the cell culture experiments. HAP and $Ca(OH)_2$ (Merck), both at the concentrations of 40 mg/mL were also extracted in the cell culture medium. The control CM was complete RPMI medium without the test samples.

Cytotoxicity assays

The cytotoxicity tests were performed *in vitro* by using a direct contact method of the discs samples with the subconfluent monolayer of L929 cells or indirectly by examining the metabolic activity of L929 cells in the presence of different dilutions of CM according to the ISO-10993-5 guideline [14]. The L929 cells are a mouse fibroblast cell line (ADCC collection, Rockville, MA, USA).

In the direct assay, L929 cells were cultivated in complete RPMI medium in 6-well plates (Sarstedt, Germany) until reaching about 80% confluency. The Ti6Al4V alloys, coated or uncoated as well as positive and negative control samples were placed into the centre of the wells and cultivated for 24 hours in an incubator with 5% CO_2. The surface of the test samples/volume of medium was 1 cm^2/mL. The cells without the test samples served as the negative control. All cultures were done in triplicates. After cultivation, the cultures were examined under the invert microscope (IX51 Inverted Microscope, Olympus) at 10 and 20x magnifications. The quality of cell monolayer was analysed in the proximity and at the distance from the samples. Confluences of cell growth indicated the absence of cytotoxicity while the rounding, vacuolization and detachment of cells indicated the existence of cytopathic effects of the samples. After the microscopic examination, the samples were removed and detached from the plastic surface by using 0.25% of trypsin dissolved in the serum free RPMI medium with addition of 0.02% ethylenediamine tetracetic acid (EDTA). The viability of L929 cells was determined by using 1% Trypan blue. The viability was calculated by subtracting the percent of dead (Trypan blue$^+$ cells) from 100%.

In the experiments with CM, the L929 cells were cultivated in 96-well plates in complete RPMI medium (1 x 10^4/well; volume 200 µL) overnight in sixplicates. After that, the medium was carefully removed and replaced with different dilutions of CM and the cells were cultivated for another 24 hours as described for direct assay. In the experiments where the effect of Ca in CM was investigated, the cultures were treated with nifelate (10 µg/mL), prior to placing CM in the cell cultures. The triplicates of cultures were used for the MTT test, whereas other triplicates were used for the detection of necrosis and apoptosis, respectively.

MTT assay

Metabolic activity was assessed by performing the assay based on mitochondrial succinate dehydrogenase ability to reduce 3- [4,5 dimethyl-thiazol-2-lyl]-2.5 diphenyl tetrazolium bromide (MTT) into the water-insoluble blue Formazan product. This reaction is directly proportional to the cell survival *in vitro* [15]. The L929 cells (1 x 10^4) were cultivated in presence of CM as described above. After cultivation, the medium was carefully removed and filled with new complete culture medium without phenol red (100 µL) in which 0.1mg/ml of MTT was dissolved. The cells were then cultivated for 4 h. After that, 100 µL of 10% sodium dodecyl sulphate (SDS) in hydrochloric acid solution 0.01 mol/L (0.01M HCl) (Serva) was added and cells were cultivated additionally for 18 h.

Formation of formazan was detected in cultures by reading the optical density (OD) of samples at 570 nm (Behring ELISA Processor II, Heidelberg, Germany). The test results were presented as the percentage of the metabolic activity of cells in the culture with the analysed samples in comparison with the metabolic activity of control, non-treated cells. Metabolic activity was calculated as follows: Metabolic activity (%) = [(OD of cells cultivated with test samples – OD of test samples cultivated without cells) / (OD of cells cultivated alone - OD of control medium)] x 100.

Apoptosis and necrosis assays

After 24 hours of cultivation in CM, apoptosis of L929 cells was determined by a morphological method. The cells were detached from the plastic substrate by trypsine and stained with the Turk solution, as we have already originally described [16]. The apoptotic cells were identified on the basis of their homogeneously stained, condensed nuclei. The number of totally calculated cells/sample was 500. The results were expressed as percentages of apoptotic cells.

Necrosis was detected after staining of the L929 cells with propidium iodide (PI) (Sigma) and the subsequent analysis of cells by the flow cytometry using the Partec Cube 6 flow cytometer. For this purpose, the L929 cells were collected from the wells after trypsinization, washed with phosphate buffered saline (PBS) and incubated with 500 µLof PI (10 µg/mL) dissolved in PBS. The labeled (necrotic) cells were analyzed immediately after staining. The results were expressed as percentages of necrotic cells by analysing 10.000 cells/sample.

Proliferation assay

The proliferative activity of the cells was studied by using a [^{3}H]-thymidine incorporation assay. The L929 cells (1 x 10^4) were plated in 96-well plates in triplicates and incubated overnight. After that, the medium was carefully removed and replaced with different dilutions of CM and the cells were cultivated for 24 hours, followed by a pulse with [^{3}H]-thymidine (1 µCi/well, Amersham, Books, UK) for another 8 hours. After that, the labelling fluids were removed and the cells were detached with 0.25% trypsine. The released radioactivity was measured in the β- liquid scintillation counter (LKB-1219 Rackbeta, Finland). The results are expressed as count per minute (cpm). The relative proliferation was determined by comparing cpm of CM cultures with cpm of cultures with the control medium used as 100%.

Statistical analysis

All experiments were carried out at least three times. The values were expressed as the mean ± standard deviation (SD). The differences between the samples were tested using one-way ANOVA with the Bonferoni post-test. The differences at $p < 0.05$ were considered as statistically significant.

Results

Cytotoxicity of HAP coated Ti6Al4V alloy samples

The first aim of this study was to examine the cytotoxicity of HAP coated Ti6Al4V alloy samples by using a direct assay on the L929 cells according to the ISO 10993-5 guideline. Three Ti6Al4V samples were tested: uncoated Ti6Al4V, one side, and two sides HAP coated Ti6Al4V disc samples. Cytotoxic Ni discs were used as the positive control. Negative controls were polystyrene disks and the L929 cells without any test materials. As presented in Figure 1, no significant changes in the cell morphology and morphological signs of cytotoxicity were observed around the Ti6Al4V samples and negative control samples compared to the control cells. The cells in culture with the positive control samples showed clear signs of cytotoxicity manifested as rounding, deadherence, necrosis and completely inhibited growth of L929 cells. No significant signs of cytotoxicity, except slight inhibition of growth, were observed around the one side coated Ti6Al4V samples. However, the morphological signs of cytotoxicity were visible around the two sides HAP coated Ti6Al4V discs, whereas the distant cells appeared healthy. When the test discs were removed, the cytotoxicity signs were noticed under all test samples, except under the negative, polystyrene controls.

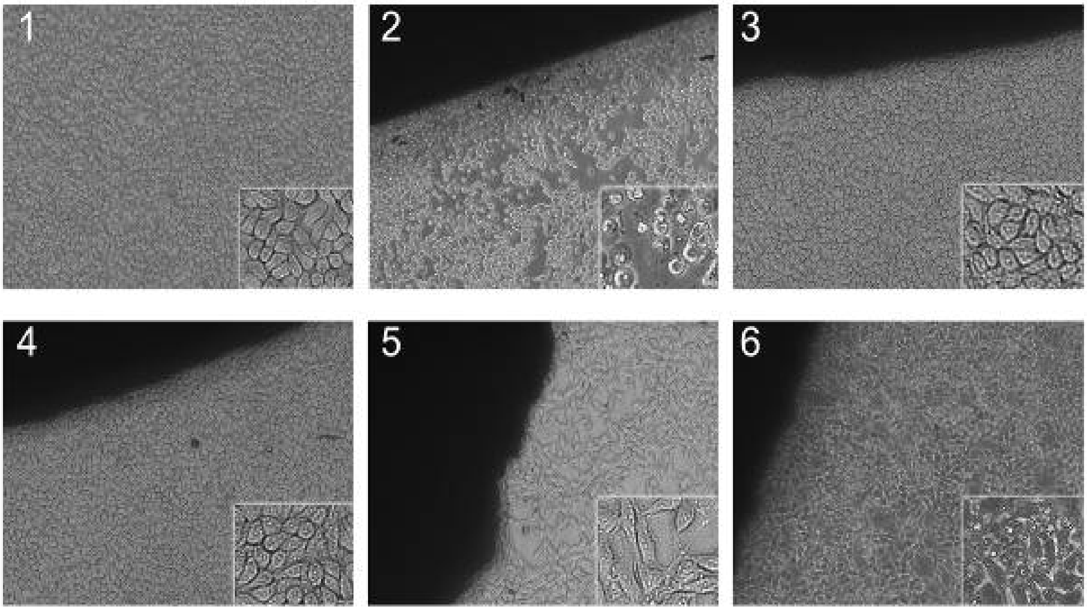

Fig. 1 – Morphological appearance of L929 cells monolayers in culture with different test samples. 1) Negative control (cells alone); 2) Positive control (nickel disc); 3) Negative control (polystyrene disc); 4) Ti6Al4V uncoated disc; 5) One side hydroxyapatite (HAP) coated Ti6Al4V disc; 6) Two sides HAP coated Ti6Al4V disc. (Magnification: x100; Inserts x 300).

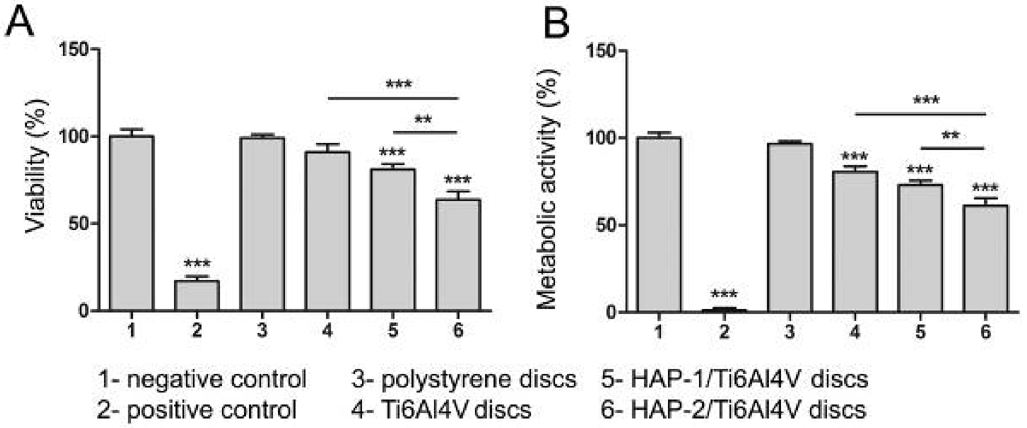

Fig. 2 – The effect of uncoated and HAP coated Ti6Al4V alloys and control samples on viability (A) and metabolic activity (B) of L929 cells.
The test samples were incubated with the L929 cells for 24 hours as described in methods. Viability was determined after removal of the samples. Metabolic activity was determined after the treatment of L929 cells with the conditioning medium (CM) of test samples by using the cell viability (MTT) assay. The results are presented as relative values (mean ± standard deviation (SD) of triplicates) compared to the negative control (cells cultivated alone) values used as 100%.
****-$p < 0.01$; ***-$p < 0.005$ compared to the negative control or to the corresponding samples, as indicated by bars**
HAP-1/HAP-2: hydroxyapatite coated on one or two sides of Ti6Al4V alloy, respectively.

These morphological observations were confirmed by using the quantitative viability assay, based on the calculation of Trypan blue positive (dead) cells (Figure 2A). The differences between the two sides HAP/Ti6Al4V and one side HAP/Ti6Al4V samples as well as between the two sides HAP/Ti6Al4V and uncoated Ti6Al4V samples, respectively, were statistically significant ($p < 0.005$). To examine whether the observed cytotoxicity was due to the released toxic products from the HAP/ Ti6Al4V alloys, CM prepared after a 24-hour conditioning of the samples in the culture medium were used. The results presented in Figure 2B showed the same pattern of cytotoxicity, except that the level of cytotoxicity was slightly higher compared to the viability assay. Based on the ISO 10993-5 criteria, the uncoated Ti6Al4V alloy and one – side HAP/Ti6Al4V alloy were classified as non–cytotoxic (reduction of viability less than 30%), whereas the two sides HAP/Ti6Al4V alloy was classified as slight–moderate cytotoxic (reduction of viability by 30%–55%).

Both apoptosis and necrosis are involved in cell death induced by CM of the HAP/Ti6Al4V alloy

The additional tests were performed to evaluate the mode of cytotoxicity: PI staining to evaluate necrosis; Türk staining to evaluate apoptosis and [^{3}H]-thymidine incorporation assay as an indicator of cell proliferation. Figure 3A showed that undiluted CM of Ti6Al4V alloys (uncoated, one side – and two sides HAP coated) induced necrosis of L929 cells. Necrosis was the highest using CM prepared from the two sides HAP/Ti6Al4V alloys and was dilution dependent. It is interesting that the lower concentrations of HAP/Ti6Al4V CM induced higher degree of apoptosis that the higher ones (Figure 3B). The observed necrosis/apoptosis results were in agreement with the results obtained in the proliferation assay. As expected, the inhibition of [^{3}H] - thymidine incorporation by the L929 cells was the highest using CM of two sides HAP/Ti6Al4V samples. All three tested Ti6Al4V CM samples showed the higher degree of inhibition of cellular proliferation compared to the level of cytotoxicity obtained by using the necrosis/apoptosis assays (Figure 3C).

Conditioning decreases the cytotoxicity of HAP/Ti6Al4V samples

Next, we examined whether the conditioning modified the cytotoxicity of HAP/Ti6Al4V alloys. In this context, the two sides coated HAP/Ti6Al4V discs were conditioned for 24 hours and then tested in the direct cytotoxicity assay. At the same time the 24 hour CM, prepared from already conditioned HAP/Ti6Al4V alloys, was tested by the MTT. The same procedures were applied for the uncoated Ti6Al4V and control samples. As presented in Figures 4A and 4B, both the direct and indirect assays clearly showed that conditioning significantly decreased the cytotoxic effect of HAP/Ti6Al4V alloys. The cytotoxicity of two sides HAP/Ti6Al4V alloys determined by the MTT assays (initial 48.2 ± 4.73) was decreased after conditioning (23.5 ± 3.0) to the level of accepted cytotoxicity according to the ISO 10993-5 criteria ($p < 0.005$). In contrast, the cytotoxicity of uncoated Ti6Al4V as well as Ni discs and their CM were not significantly modified. No further reduction of cytotoxicity was observed after the additional conditioning of samples for 7 days (Figures 4C and 4D).

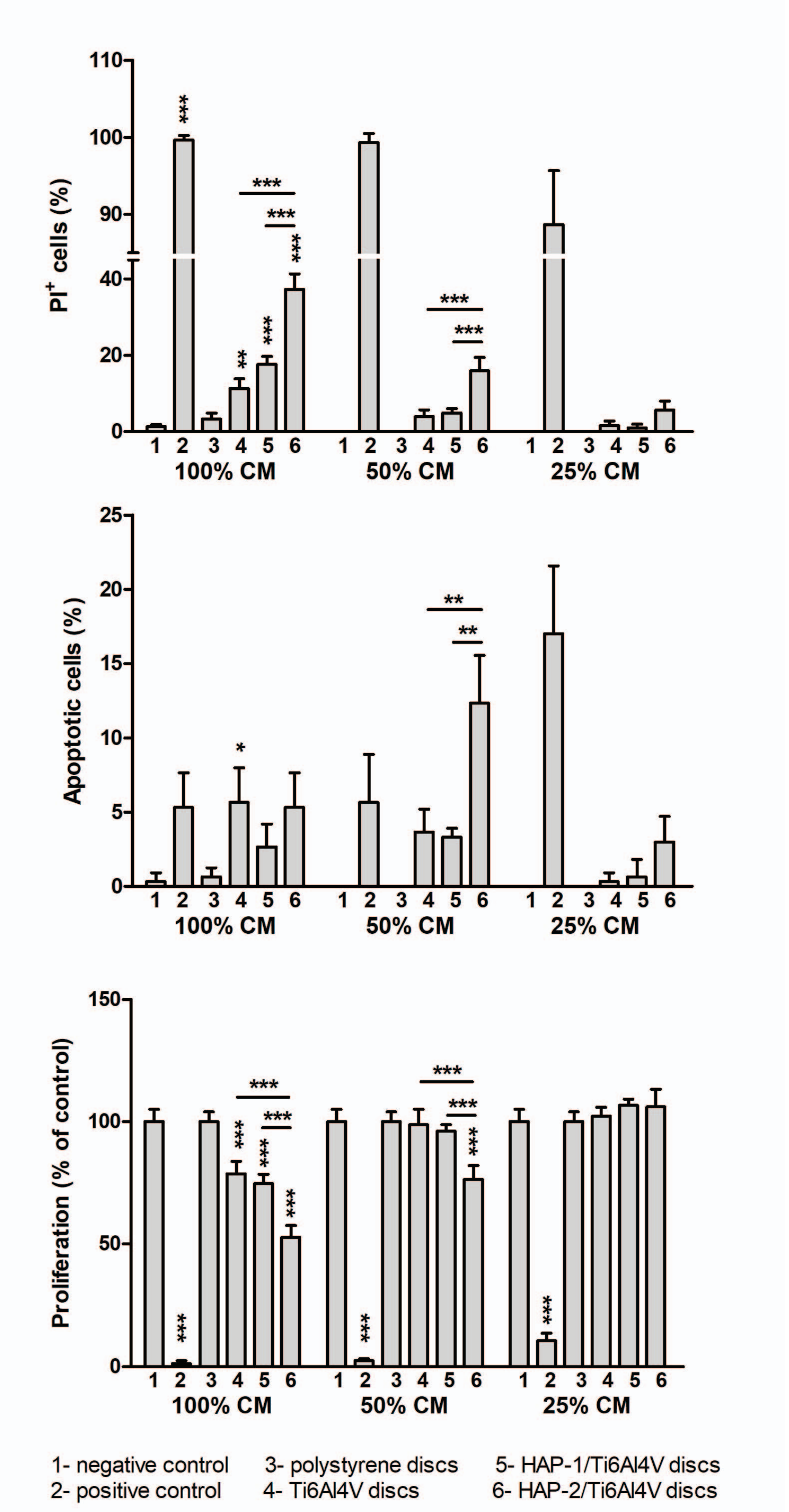

Fig. 3 – The effect of conditioned medium (CM) of Ti6Al4V alloys and control samples on necrosis (A), apoptosis (B) and proliferation (C) of L929 cells.

The L929 cells were cultivated with undiluted (100%), 50% and 25% conditioning medium (CM) for 24 hours. After that necrosis, apoptosis and proliferation were determined as described in methods. Results are presented as percentages of necrotic (propidium iodide – PI+ cells), percentage of apoptotic cells or as relative proliferation compared to the negative control used as 100% [all as mean ± standard deviation (SD) of triplicates].

***-$p < 0.05$; **-$p < 0.01$; ***-$p < 0.005$ compared to the negative control or to corresponding samples, as indicated by bars. HAP-1/HAP-2: hydroxyapatite coated on one or two sides of Ti6Al4V alloy, respectively.**

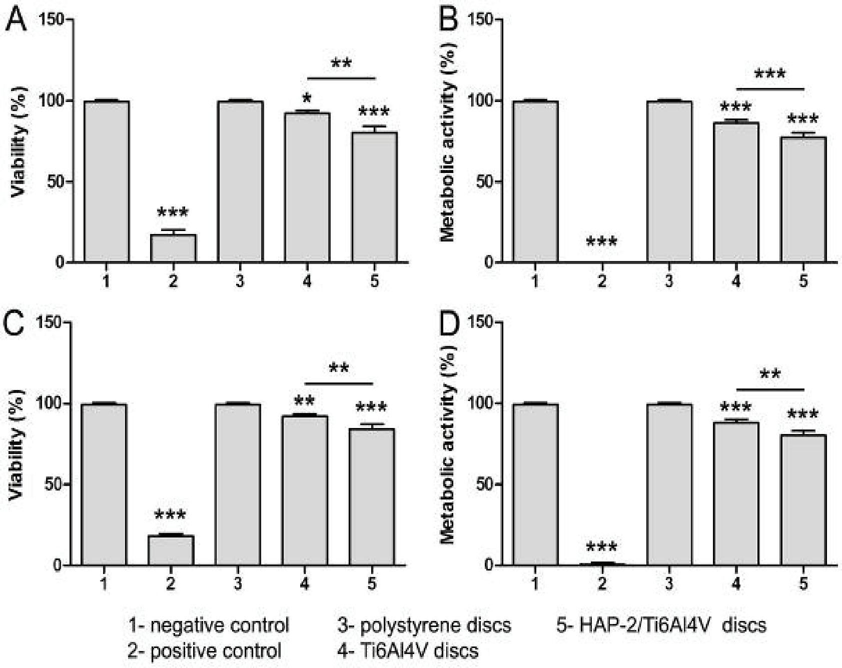

Fig. 4 – The effect of conditioned Ti6Al4V alloys and CM of the conditioned Ti6Al4V alloys on viability (A, C) and metabolic activity (B, D) of L929 cells.

The test samples were conditioned in RPMI culture medium for 24 hours (A, B) or 7 days (C, D) as described, and used in the direct assays with the L929 cells to assess the viability. CM were prepared by incubating the conditioned samples for 24 hours. Such CM were cultivated with L929 cells for 24 hours and then the metabolic activity of the cells was examined.
***-$p < 0.05$; **$p < 0.01$; ***-$p < 0.005$ compared to the negative control or to corresponding samples, as indicated by bars**
HAP-1/HAP-2: hydroxyapatite coated on one or two sides of Ti6Al4V alloy, respectively.

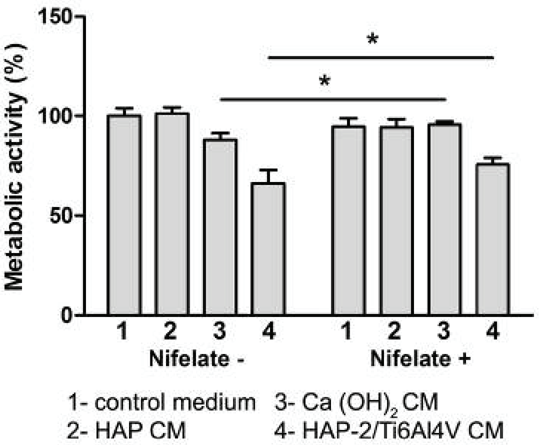

Fig. 5 – The effect of nifelate on the metabolic activity of L929 cells treated with different conditioned media.

The L929 cells were treated with CM prepared from the HAP, $Ca(OH)_2$ or two sides HAP-coated Ti6Al4V alloy in presence or absence of nifelate (10 µg/mL) for 24 hours and after that the metabolic activity of the cells was determined by cell viability (MTT) assay as described in methods.
The results are presented as relative metabolic activity [mean ± standard deviation (SD); n = 3] compared to the values of control cells.
***-$p < 0.05$ compared to corresponding samples as indicated by bars.**

The role of Ca in cytotoxicity of the HAP coated Ti6Al4V alloy

The final aim of this work was to examine the possible role of Ca ions in the HAP/Ti6Al4V cytotoxicity, based on the previous results which showed that this process depended on the amount of HAP used for coating. The estimated amount of HAP on the two sides coated Ti6Al4V alloy was 40 mg. Therefore, this amount of HAP was conditioned in the same volume of complete RPMI medium as used for the Ti alloy conditioning. As shown in Figure 5, CM from HAP was not cytotoxic, indicating that released HAP from the alloy was probably not a cause of cytotoxicity. Since HAP is hardly soluble in water solutions, we tested CM prepared from Ca(OH)2 which is more soluble under the same conditions. We showed that CM from $Ca(OH)_2$ was slightly cytotoxic, but much lower than CM of HAP/ Ti6Al4V samples.

Finally, we used nifelate (Ca channel blocker) in the assay with CM. Nifelate abrogated completely the cytotoxic effect of $Ca(OH)_2$ CM, but only slightly reduced the cytotoxicity of HAP/Ti6Al4V CM.

Discussion

Modification of surface of the Ti alloys, which are used as implants in dentistry and orthopaedic surgery, can lead to favourable bone regeneration and integrity between the bone tissue and implant surface [17]. In this context, HAP has been widely used due to its good biocompatibility. However, the bioactivity of HAP coatings *in vivo* largely depends on the applied method [18]. The plasma-sprayed HAP coatings frequently show a large variation in the quality of the HAP layer including poor coating – metal adhesion strength, non-uniformity in coating thickness and density, as well as changes in the structural properties during the coating process [18]. The process of plasma spraying can also influence the change from crystal to amorphous form in the HAP phase composition [19].

To improve most of these parameters, our research group used an innovative plasma jet method, with high electric energy input, for the HAP coating on high purity Ti substrate [13]. The procedure enabled extraordinarily high adhesion strengths of the obtained coatings, showing a very rough surface with micro and nano patterns. Therefore, the same method was applied in this study for the HAP coating of Ti6Al4V alloy. Although there was no detailed microstructural analysis of the Ti6Al4V coating, during cytotoxicity investigation we found that HAP layer was quite stable, thus demonstrating the paramount importance for oseointegration.

The biocompatibility studies start from the cytotoxicity assay *in vitro* [14] and this was the principal goal of this work. Our results showed that all examined Ti6Al4V samples exerted a certain degree of cytotoxicity. The cytotoxicity of uncoated and one side coated Ti6Al4V samples was acceptable according to the current ISO 10993-5 criteria [14], since the degree of cytotoxicity did not exceed 30%. The cytotoxicity of two sides HAP coated Ti6Al4V samples was in the range of 30%–55% and because of that they were classified as slight-moderate cytotoxic. The cytotoxicity was verified according to the reduction of cell viability (MTT and morphological assays), decrease of cellular proliferation and induction both of necrosis and apoptosis of L929 cells.

It is interesting that we observed a lower level of apoptosis when CM, prepared from the two sides HAP Ti6Al4V alloys, was tested in higher concentrations. In contrast, necrosis of L929 cells was concentration dependent. This phenomenon could be explained by the fact that cells triggered to undergo apoptosis will die by necrosis when the intracellular energy level is low, such as adenosine triphosphate (ATP) depletion [20]. Based on this concept, it can be postulated that the higher concentrations of toxic compounds in the medium extract blocked the mitochondrial or glycolytic ATP generation and caused necrosis. When their concentrations are lower the threshold ATP concentrations are sufficient to execute the apoptotic programme.

What could be the mechanisms involved in cytotoxicity? It is obvious that they are different depending on the used samples. A slight cytotoxicity of uncoated Ti6Al4V alloy, which was already described in literature [21] could be due to the release of cytotoxic Ti, aluminium (Al) and vanadium (V) ions [21, 22]. The release of these ions is usually bellow cytotoxic concentrations, due to the formation of a Ti-oxide protective layer [21–23], but they could act synergistically at the subtoxic concentrations. These ions could be also released from the HAP coated Ti6Al4V discs.

However, a certain degree of cytotoxicity might be caused by the HAP coatings. Crystalline HAP is not cytotoxic because it is hardly soluble in water solutions. We also confirmed in this study that CM prepared from HAP did not modify the metabolic activity of L929 cells. The nanostructure forms of HAP show some degree of cytotoxicity as demonstrated on HepG2 cells [24] due to the induction of oxidative stress and subsequent cytopathic effects through both necrotic and apoptotic mechanisms.

We hypothesize that the most pronounced cytotoxic effect on the L929 cells observed in this study by the two sides HAP coated Ti6Al4V was induced by the amorphous forms of the coating layer, including calcium oxide (CaO), three- and tetracalcium phosphate. These phases, which were developed during the applied plasma spraying procedure [13, 25] are soluble in water solutions [26]. Of them, CaO is the most soluble and non-biocompatible compound [26–28]. CaO forms $Ca(OH)_2$ in water. Therefore, we tested whether CM prepared from $Ca(OH)_2$ suspension was cytotoxic. The answer was yes, similarly as described for the $Ca(OH)_2$ nanoparticles [29] , but the degree of cytotoxicity was lower compared to CM from the HAP/Ti6Al4V alloys.

The hypothesis that soluble components of HAP coating could be responsible for the obtained results is in line with the observations that conditioning of the two sides HAP/Ti6Al4V alloy significantly reduced its cytotoxic effect up to the non-cytotoxic level according to the ISO 10993-5. Based on this original finding, we can suggest using a kind of conditioning procedure for the HAP coated metal alloys before their implantation *in vivo*, as a helpful method to reduce the cytotoxicity. However, to make this presumption more relevant, it is necessary to determine the concentrations of Ca released in the culture media during conditioning simultaneously with the characterization of HAP coating by Auger microscopy, as well as to test other water solutions instead of culture medium.

To check the possible effect of soluble Ca for the observed cytotoxicity of CM prepared from the HAP-Ti6Al4V alloys, we blocked the Ca channels by nifelate. We showed that the cytotoxicity under such experimental conditions was only slightly diminished, indicating that Ca ions could not be a key factor influencing the cytotoxicity. Other mechanisms could be related to the ingestions of micro or nano HAP particles which might be released in CM. Such particles were visible around disc samples in the direct cytotoxicity assay. Recent studies showed that nano-HAP was cytotoxic due to the interference of ingested particles with different signalling mechanisms including those related to cell proliferation/death[30]. It is also possible that cytotoxicity of HAP/Ti6Al4V CM could be due to the synergism between released metal ions and the component of amorphous HAP phases, either in their soluble or particulate forms. To make this conclusion more relevant, elemental analysis of these components will be necessary and this investigation is in progress.

Conclusion

This study shows that the HAP coatings obtained by an innovative plasma jet deposition on the Ti6Al4V alloy enables good adhesion stability of the coated layer. However, when both sides of disc samples of the Ti6Al4V alloy are coated, the cytotoxicity of target L929 cells was enhanced. The cytotoxicity was reduced to the non-cytotoxic level by conditioning of the HAP/Ti6Al4V alloy in culture medium for 24 hours, most probably due to the removal of the amorphous phase of HAP. Therefore, a conditioning procedure could be helpful if applied before the implantation of HAP coated metal alloys *in vivo*.

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