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Characterization of Rabbit Mesenchymal Cell Attachment on Calcium Phosphate Surface

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***Abstract* – In the current study, the effect of three different treated surfaces of hydroxyapatite and β-tricalcium phosphate on mesenchymal cell attachment has been investigated. Calcium phosphate powders have been synthesized, uniaxially pressed, polished and sintered. Mesenchymal cells have been seeded onto unpolished, polished and polished-thermally etched ceramic samples. The ceramic samples have been characterized by XRD, FTIR and SEM. Results have shown that the best cell attachment and morphology are on the unpolished surface indicating that relatively rough surface is better for cell application.**

***Keywords* – Hydroxyapatite, implant surface, mesenchymal cells, β-tricalcium phosphate.**

I. Introduction

Calcium phosphate (CaP) bioceramics, such as hydroxyapatite (Hap) and β-tricalcium phosphate (β-TCP), are important biomaterials for dental, craniofacial and orthopedic repairs due to their similarity to bone mineral component and bone bonding ability to form a functional interface. Many scientific studies are devoted to the investigation of coatings and their surface of metallic implants [1–4]. The most popular materials applied as metal implant coatings are calcium phosphates due to their ability to form an apatite layer indicating bioactive and osteoconductive properties [5–6]. The characteristics of surface of any biomaterial are essential for protein adsorption after a surgical procedure and subsequently cell attachment and proliferation. In fact, there are many methods for surface treatment and modification. The ion implantation [7], powder abrasive treatment [8], laser irradiation [9], plasma spraying [10] and modification with nanoparticles [11] are named as the most popular approaches.

The bone marrow stromal cells or mesenchymal stem cells (MSCs) have been shown to differentiate into bone, as well as cartilage and fat cells [12], which makes them ideal candidates for developing bone tissue-engineered constructs. It is well known that CaP materials promote MSCs differentiation down the osteogenic lineage [13–15] and that surface topography and particle size have an effect on cell proliferation and differentiation [16–18].

Hap and β-TCP ceramics are popular scaffold materials in tissue engineering for stem cell seeding. CaP ceramics, seeded with stem cells, are a promising approach for better bone tissue ingrowth. Increasing cell seeding efficiency in a tissue engineering construct, it is possible to enhance a cellular activity and tissue formation as well.

In the current study, different treated Hap and β-TCP ceramic surfaces were used to investigate rabbit mesenchymal stem cell attachment. Three various, but simple surface treatment technologies (sintering, polishing and polishing-thermally etching) were used. Up to date there is no study, regarding the effects of Hap and β-TCP surface morphology, treated by polishing, sintering and polishing-thermally etching, on cellular response. The aim of the research is to identify morphology of the implant surfaces, which could enhance cell attachment. The understanding of impact of surface properties on cells, proteins and tissue response could give knowledge for development of dental and orthopaedic implants.

II. Materials and Methods

A. Preparation of Calcium Phosphates Powders

Calcium phosphate powders were prepared by a wet chemical precipitation method using calcium oxide (*Fluka, Germany*) and ortophosphoric acid (*Sigma-Aldrich, Germany*) as raw materials. The process can be described by the following reactions:

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad (1)$$

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O \quad (2)$$

This method has been characterized by a simple process, low cost, easy application in industrial production and water is the only by-product. The method is found to be highly dependent on the selected technological parameters, such as reagents, impurity content, concentration of reagents, mixing conditions, pH and temperature. The raw material was calcined at 1000 °C for 1 h to obtain pure CaO, then distilled water was added to gain $Ca(OH)_2$ suspension with concentration 0.15 M (1). The precipitation reaction was carried out at 45 °C for Hap or ambient temperature for β-TCP production. The 2 M phosphoric acid solution was added slowly into the calcium hydroxide solution (2). The mixture was stirred for 1 h, after the end pH value (8.7 for Hap or 6.0 for β-TCP) was obtained, then aged for 20 h at room temperature. Lower synthesis temperature and acidic pH values promote formation of β-TCP phase, but slightly alkaline pH and higher temperature promote formation of Hap phase. Varying these parameters, it is possible to obtain a calcium phosphate product with diverse phase composition. Thereafter filtered precipitates were dried at 105 °C for 24 h. The as-synthesized powder was heat treaded at 1100 °C for 1 h to establish phase and chemical purity.

B. Examination of Calcium Phosphate Powders

The phase composition and chemical purity of Hap and β-TCP powders were investigated using X-ray diffractometry (XRD) and Fourier transform infrared spectroscopy (FTIR).

XRD analysis was carried out with *PANalytical X'Pert Pro*, Cu Kα1, 40 kV, 30 mA. FTIR analysis was performed with *Varian Scimitar 800* in the wavenumber range of 4000–400 cm^{-1}.

C. Preparation of Ceramic Samples

Obtained as-synthesized powders were uniaxially pressed into pellets (d = 10 mm, H = 3 mm). All samples were sintered at 1100 ºC for 1 hour. Unpolished, polished and thermally etched samples were used for investigation of stem cell attachment. Polished ceramic samples were prepared by polishing with 6 μm, 1 μm, 0,25 μm diamond paste and cleaned in the ultrasonic bath using deionized water for 1 min. Thermal etching was carried out at 1000 ºC for 4 min, to reveal the grain boundaries of matrix grains after polishing. Hap and β-TCP pellets were steam sterilized in an autoclave at 121 °C for 30 min.

D. Examination of Ceramic Samples

For the scanning electron microscopy (SEM) samples with a thin layer of gold was prepared using gold sputter coater (Emitech K550X, QUORUM TECHNOLOGIES Company) with a sputtering rate of 7 nm/min applying 25 mA current. The total gold coating thickness was approximately 14 nm. Micrographs were obtained using Mira\\LMU Schottky-Emission electron gun SEM (TESCAN Company) at an accelerating voltage of 15 kV and magnification of 3,000 x. The double detector system was used.

Cells were fixed in 2.5 % glutaraldehyde and dehydrated with increasing concentrations of acetone (50 %, 60 %, 70 %, 80 %, 90 %) for 10 minutes each concentration, then samples were dried in critical point dryer (Polaron, OM-E3000, QUORUM TECHNOLOGIES Company) for 1 hour in CO_2 medium.

The density and porosity of the samples were determined using the Archimedes' method.

E. Mesenchymal Cell Isolation and Culture

The rabbit mesenchymal cells were isolated using enzymatic digestion with collagenase type XI (Sigma) and seeded for expansion in Dulbecco's Modified Eagle Medium (DMEM)/10 % fetal bovine serum (FBS) in standard culture conditions (37 °C, 95 % relative humidity, and 5 % CO_2). Cells were passaged by trypsinizing (0.05 % trypsin/ ethylenediaminetetraacetic acid (EDTA)) and subcultured at a density of 4000 cells/cm^2 – 5000 cells/cm^2. For all experiments cells between the third and fifth passage were used. On average 70000 cells were seeded onto the Hap and β-TCP ceramic pellets one day prior further use.

III. Results and Discussion

The X-ray diffraction patterns of the synthetic calcium phosphate powders show the phase composition after thermal treatment in 1100 °C for 1 hour (Fig. 1). The sharp peaks indicate well crystalline calcium phosphate powders. In Fig. 1 A, HAp phase is represented, and in Fig. 1 B, β-TCP phase can be seen. All maximums, which are shown in the diffractogramms, correspond to pure HAp and pure β-TCP phases. The crystalline phases detected in the patterns were identified according to standard patterns from the ICDD – PDF database (No. 9-432 for HAp and No. 55-898 for β-TCP).

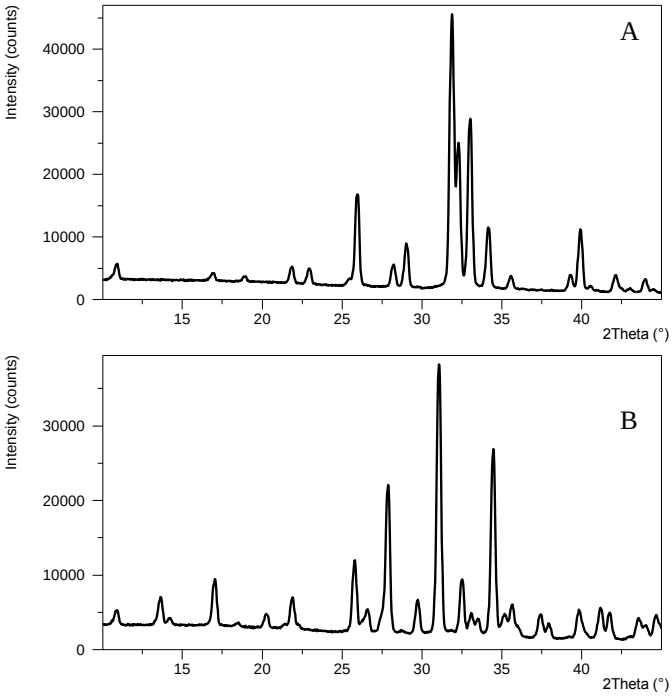

Fig. 1. X-ray diffractometry patterns: A – HAp phase, B – β-TCP phase.

FTIR analysis shows the characteristic vibrations of chemical bands of HAp (Fig. 2. A) and β-TCP (Fig. 2. B).

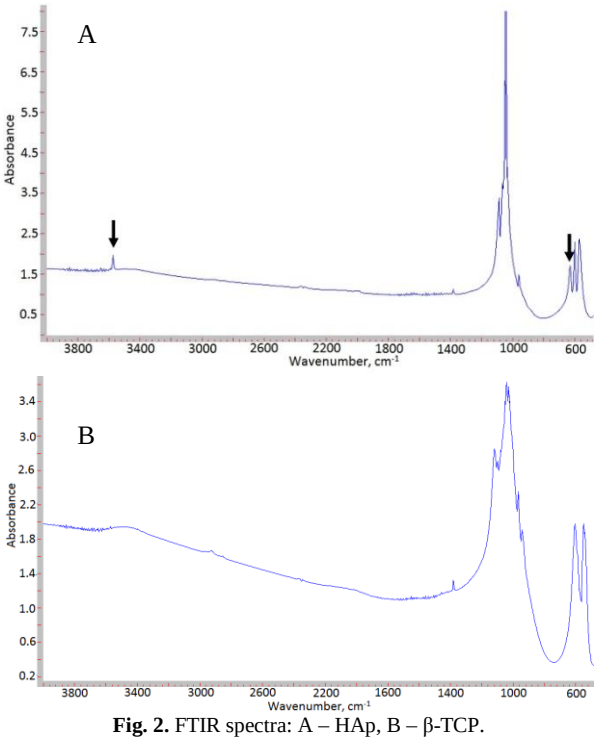

Fig. 2. FTIR spectra: A – HAp, B – β-TCP.

FTIR spectra show vibration modes of PO_4^{-3} ions in the wavenumber range of 550 cm^{-1} – 600 cm^{-1}, 962 cm^{-1}, 1020 cm^{-1} – 1120 cm^{-1}, those are typical for HAp and β-TCP. HAp phase can be confirmed with peaks indicating OH^- functional groups located at wavenumbers of 640 cm^{-1} and 3571 cm^{-1}, denoted with

arrows (Fig. 2 A). On the contrary, there is no evidence of OH^- groups in Fig. 2. B that proves the existence of pure β-TCP phase; in addition, the broadening of stretching mode of PO_4^{3-} group is a typical indication of formation of β-TCP.

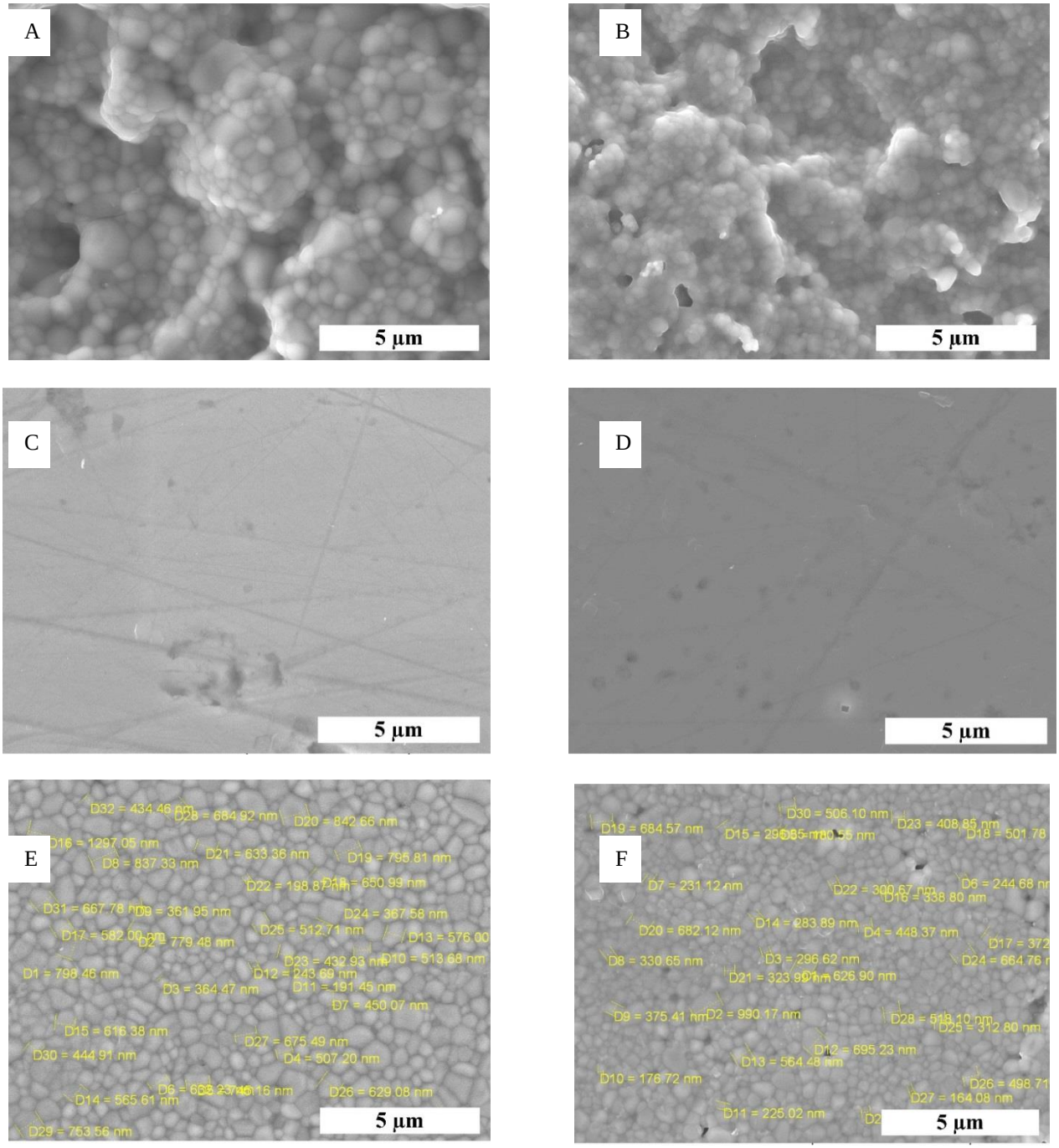

Fig. 3. SEM micrographs: A – unpolished β-TCP, B – unpolished HAp, C – polished β-TCP, D – polished HAp, E – polished and thermally etched β-TCP, F – polished and thermally etched HAp.

The microstructural comparison of the prepared samples is presented in Fig. 3. Sintered and unpolished surfaces of β-TCP and HAp have similar morphology. It is rounded and relatively rough with clearly visible grains, see Fig. 3 A and B micrographs. SEM images C and D demonstrate polished surfaces of both ceramics. There are no significant differences between β-TCP and HAp surfaces. There are some grooves resulting from polishing procedure. However, the microstructural evaluation of polished and then thermally etched samples (Fig. 3. E and F) reveals the structure of grains. Results indicate that β-TCP ceramic (Fig. 3. E) has larger grains (D_{Av} = 587 nm) compared to HAp ceramic (D_{Av} = 566 nm) (Fig. 3. F), which is according to literature [19]. In E and F images, it can be seen that β-TCP microstructure is denser, while HAp microstructure has some micropores. The density of β-TCP and HAp ceramics is 2.6 g/cm^3 and 2.2 g/cm^3, respectively. Measurement and calculation of porosity indicates that total porosity is in range from 15 % to 17 % for β-TCP and 27 % to 31 % for HAp. The density and porosity differences could be explained due to

sintering temperature. β-TCP has a lower sintering temperature than HAp that agrees with the studies of other authors [20].

TABLE 1

POROSITY AND DENSITY OF β-TCP AND HAP SAMPLES

	Density [g/cm^3]	P_{open} [%]	P_{closed} [%]	P_{total} [%]
β-TCP-*np*	2.60 ± 0.01	14.2 ± 0.4	3.3 ± 0.2	17.5 ± 0.4
β-TCP-*p*	2.64 ± 0.04	10.1 ± 2.2	4.9 ± 1.0	14.9 ± 1.3
β-TCP-*te*	2.61 ± 0.01	13.5 ± 0.5	2.9 ± 0.2	16.4 ± 0.4
HAp-*np*	2.23 ± 0.07	26.9 ± 2.2	4.0 ± 0.5	31.1 ± 2.1
HAp-*p*	2.30 ± 0.02	22.3 ± 0.3	4.6 ± 0.2	26.9 ± 0.1
HAp-*te*	2.25 ± 0.01	24.2 ± 0.6	5.0 ± 0.7	29.2 ± 0.2

Abbreviations: *np* – non-polished, *p* – polished, *te* – thermally etched

All samples seeded with mesenchymal cells were examined 24 hours after cell seeding on the biomaterial surface. In all cases (Fig. 4.), morphology of cells differs. On the unpolished surface, cells exhibit an indistinct and flattened shape, while on the polished surface cells have a more rounded shape. Considering cell shape, thermally-etched samples showed a relatively worse cell vitality, compared to the cells on the unpolished and polished surfaces. SEM analysis revealed a relatively better mesenchymal cell adhesion on the hydroxyapatite samples than β-tricalcium phosphate samples. In fact, aggregates of cells were observed, see Fig. 3 G. Roohani-Esfahani et al. have investigated the effect of material surface characteristics on the mesenchymal cell proliferation and differentiation. They observed that flatter topographies enhance cell proliferation while rougher, micro-scale topographies enhance osteogenic differentiation of cells [12]. Although we could not study proliferation and differentiation as cells were observed already after 24 hours, in accordance with the reviewed authors' group, it could be concluded that the rough surface of calcium phosphates was more favourable for cells in comparison with smoother surfaces.

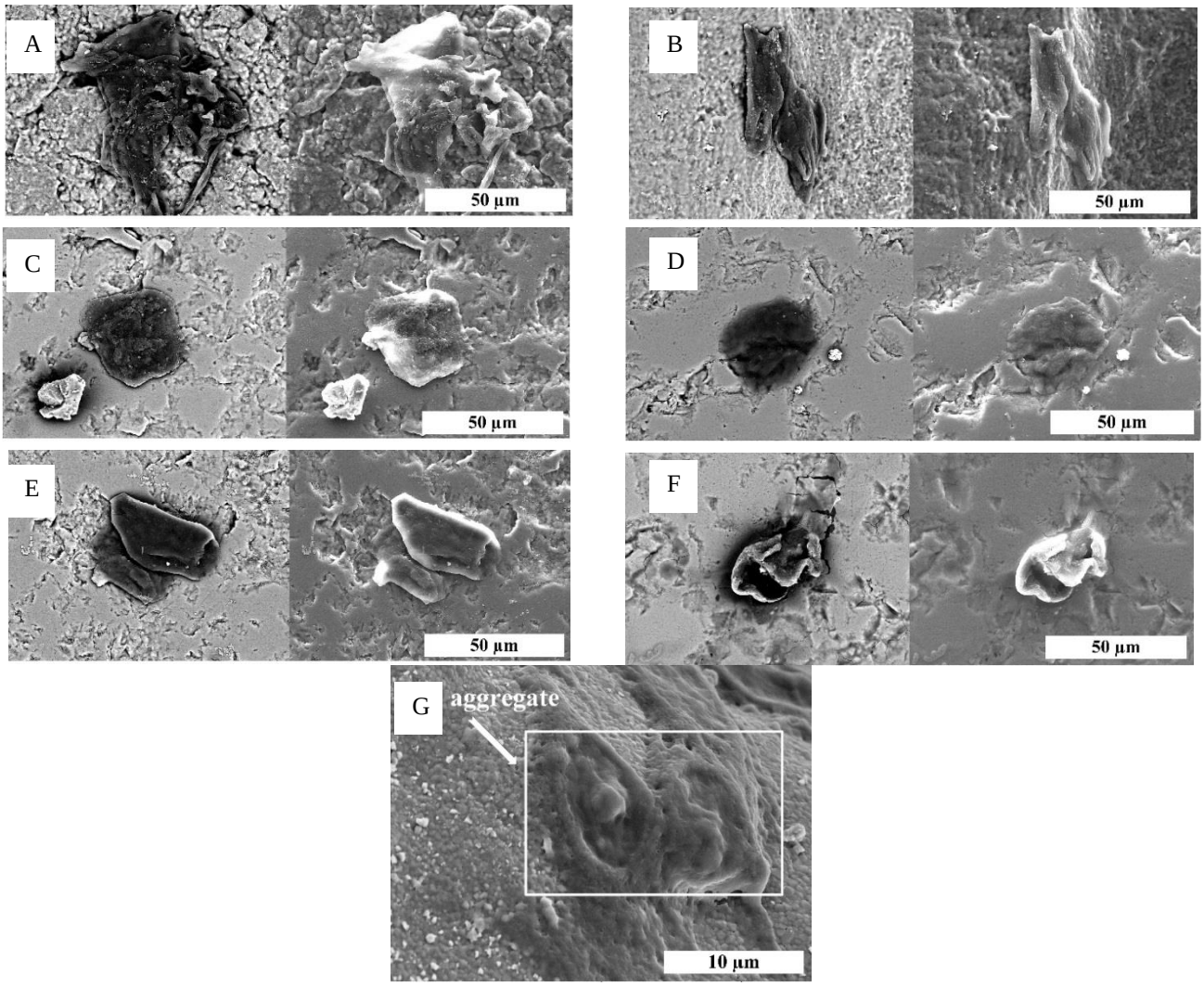

Fig. 4. SEM images of ceramic samples with mesenchymal stem cells: A – unpolished β-TCP, B – unpolished HAp, C – polished β-TCP, D – polished HAp, E – polished and thermally etched β-TCP, F – polished and thermally etched Hap, G – aggregate of cells on unpolished Hap.

IV. Conclusions

As the cells are living organisms, they should connect to each other to have a possibility of forming a new tissue [21]. Rounded cell morphology indicates that probably cells do not aim to connect to each other.

The microstructural effect of hydroxyapatite and β-tricalcium phosphate on rabbit mesenchymal stem cells has been sudied. SEM investigation revealed the differences between a surface microstructure of HAp and β-TCP, which are mainly attributed to grain size, density and porosity. β-TCP has larger grains and denser microstructure as HAp at the same thermal and surface treatment conditions.

The current study revealed that material surface features could influence attachment and morphology of cells. The smooth surfaces, in this case, polished and polished-thermally etched, were not favourable for cell attachment, while an unpolished surface was more suitable for cell application. There were no scientific differences discovered between droxyapatite and β-tricalcium phosphate phases regarding mesenchymal cell attachment and morphology, but it is an important factor that needs to be taken into account.

The future work will be devoted not only to surface topography investigation, but also to chemical factors of biomaterial that could influence cell behavior on the calcium phosphate scaffolds.

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Vita Zālīte, Marina Sokolova, Dmitrijs Jakovlevs, Kārlis Rozenbergs, Līga Bērziņa-Cimdiņa. Truša mezenhīmas šūnu piestiprināšanās raksturošana uz kalcija fosfātu virsmas

Šajā darbā tika pētīta truša mezenhīmas šūnu piestiprināšanās spēja un šūnu morfoloģija uz dažādi apstrādātām hidroksilapatīta (HAp) un β-trikalcija fosfāta (β-TCP) virsmām. Šāda pētījuma nozīmība saistās ar to, ka ir būtiski noskaidrot, kādai jābūt implanta virsmai, lai nodrošinātu veiksmīgu šūnu piestiprināšanos, kam vēlāk seko šūnu migrācija, proliferācija un diferenciācija. Zinātniskais darbs sevī ietver vairākas daļas: HAp un β-TCP pulveru sintēzi un to analīzi ar rentgenstaru difraktometriju un Furjē transformāciju infrasarkano spektroskopiju, HAp un β-TCP presētu paraugu sagatavošanu un to virsmas modificēšanu, kā arī truša mezenhīmas šūnu iegūšanu, inkubēšanu un uznešanu uz HAp un β-TCP paraugiem. HAp un β-TCP presētu paraugu virsmas ar un bez šūnām tika analizētas, izmantojot skenējošo elektronu mikroskopiju. Pētījumā tika apskatīta neapstrādāta, pulēta un pulēta-termiski kodināta HAp un β-TCP keramikas virsma. Būtiskākās keramikas mikrostruktūras atšķirības bija graudu izmērs, jo β-TCP keramikai ir zemāka saķepšanas sākuma temperatūra, kas noved pie lielāka graudu izmēra salīdzinājumā ar HAp keramiku, kas termiski apstrādāta 1100 °C temperatūrā, apstrādes laiks 1 h. Līdz ar to β-TCP keramikai ir raksturīga blīvāka mikrostruktūra. Eksperimenti ar mezenhīmas šūnām parādīja, ka tām labvēlīgāka ir neapstrādāta keramikas virsma, jo šūnu forma bija plakana un nenoteikta, kas varētu norādīt, ka šāda šūna labprātāk „komunicēs" ar citām šūnām un sāks veidot jaunus audus. Ja šūnām ir noapaļota forma, tad paredzams, ka pēc laika tās ies bojā un jaunu audu veidošanās nenotiks. Pētījums parādīja, ka mezenhīmas šūnas labprātāk piestiprinās relatīvi raupjai materiāla virsmai gan HAp, gan β-TCP keramikas gadījumā. Iegūtais rezultāts ir labvēlīgs gadījumos, kad nepieciešams izgatavot sarežģītas formas un struktūras implantus, kuriem nav iespējama virsmas apstrāde.

Вита Залите, Марина Соколова, Дмитрий Яковлевс, Карлис Розенбергс, Лига Берзиня-Цимдыня. Характеристика закрепления мезенхимальных клеток кролика на поверхности фосфата кальция

В настоящей научной работе была изучена способность прикрепления мезенхимальных клеток кролика и морфология клеток на различных поверхностях гидроксиапатита (ГАП) и трикальцийфосфата (ТКФ). Важность такого исследования связана с тем, что необходимо знать, какой должна быть поверхность имплантата для того, чтобы обеспечить успешное прикрепление клеток для дальнейшей миграции, пролиферации и дифференциации. Научная работа включает в себя несколько частей: синтез порошков гидроксиапатита и трикальцийфосфата, их анализ методом рентгеновской дифрактометрии и инфракрасной спектроскопии Фурье, подготовка прессованных образцов и модификация их поверхности, а также получение мезенхимальных клеток кролика, их инкубация и нанесение на образцы ГАП и ТКФ. Поверхность прессованных ГАП и ТКФ образцов с клетками и без клеток была проанализирована с помощью сканирующего электронного микроскопа. В процессе исследования были рассмотрены необработанные, полированные и термически - полированные травленые ГАП и ТКФ керамические поверхности. Наиболее существенным отличием микроструктуры керамики был размер зерна, так как начальная температура спекания ТКФ керамики ниже, что приводит к большим размерам зерна по сравнению с ГАП керамикой при термообработке 1100 °С (1 час). Следовательно, ТКФ керамика характеризуется плотной микроструктурой. Эксперименты с мезенхимальными клетками показали, что самая благоприятная – это необработанная керамическая поверхность, потому что форма клеток была плоской и неопределенной, что в свою очередь означает, что такая клетка предпочтет "общаться" с другими клетками и начнет строить новую ткань. Если клетки имеют округлую форму, тогда ожидается, что со временем они будут разрушены, а образования новых тканей не произойдет. Исследования показали, что мезенхимальные клетки предпочитают цепляться к относительно грубой поверхности материала – также в случае ГАП и ТКФ керамики. Полученный результат благоприятен в случае, когда необходимо получение имплантатов сложных форм и конструкций, которые не позволяют проводить обработку поверхности.