OSTEOBLASTS RESPONSE TO NOVEL CHITOSAN/AGAROSE/ HYDROXYAPATITE BONE SCAFFOLD – STUDIES ON MC3T3-E1 AND HFOB 1.19 CELLULAR MODELS

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Abstract

Since it is known that various cell lines may express different behaviours on the scaffolds surface, a comprehensive analysis using various cellular models is needed to evaluate the biomedical potential of developed biomaterials under in vitro conditions. Thus, the aim of this work was to fabricate bone scaffolds composed of a chitosan-agarose matrix reinforced with nanohydroxyapatite and compare the biological response of two cell lines, i.e. mouse calvarial preosteoblasts (MC3T3-E1 Subclone 4) and human foetal osteoblasts (hFOB 1.19). Within this study, the osteoblasts number on the scaffold surface and the osteogenic markers level produced by MC3T3-E1 and hFOB 1.19 cells were determined. Furthermore, changes in calcium and phosphorous ions concentrations in the culture media dedicated for MC3T3-E1 and hFOB 1.19 were estimated after the biomaterial incubation.

The obtained results proved that the fabricated biomaterial is characterized by biocompatibility and osteoconductivity since it favours osteoblasts attachment and growth. It also supports the production of osteogenic markers (collagen, bALP, osteocalcin) by MC3T3-E1 and hFOB 1.19 cells. Interestingly, the developed biomaterial exhibits different ion reactivity values in the two culture media dedicated for the mentioned cell lines. It was also revealed that mouse and human osteoblasts differ in the cellular response to the fabricated scaffold. Thus, the use of at least two various cellular models is recommended to carry out a reliable biological characterization of the novel biomaterial. These results demonstrate that the tested bone scaffold is a promising biomaterial for bone regeneration applications, however further biological and physicochemical experiments are essential to fully assess its biomedical potential.

Keywords: *bone tissue engineering, biocompatibility, osteoconductivity, cell growth, osteogenic differentiation*

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Introduction

Bone grafting is a routinely applied treatment in regenerative medicine. Despite the high efficiency of bone tissue transplantations, this treatment is constrained by painful procedures of tissue harvesting, donor-site morbidity, potential infections, disease transmission and anatomical limitations. Thus, whenever the application of bone grafts is impossible, tissue-engineered constructs are used in regenerative medicine [1,2]. Typical bone scaffolds applied in bone tissue engineering (BTE) are characterized by a three-dimensional (3D) structure which imitates the microstructure of natural bone. BTE involves the use of biomaterials as cellular devices or as scaffolds combined with cells, growth factors and/or drugs [3].

It is worth emphasizing that tissue-engineered constructs should trigger the right host response without side effects, e.g. chronic inflammation or immune rejection [4]. In order to assess the medical potential of fabricated scaffolds under *in vitro* conditions, the biomaterials are subjected to a comprehensive analysis using various cellular models [5,6]. The cellular response to the bone scaffolds depends on many features of the biomaterials. First of all, the architecture of scaffolds is of critical importance. Bone scaffolds should be characterized by high porosity with interconnected pore structure to ensure space for cells penetration and new vascular network formation. Moreover, the biomaterial porous structure allows nutrients and waste products diffusion and ensures good oxygenation [1,7,8]. Another critical feature is the pores size within the solid structure of the scaffold. According to the available literature, the pore size of at least 100 μm is considered crucial for bone ingrowth [7]. Furthermore, scaffolds for BTE applications should have adequate mechanical properties to withstand loads at the implantation site [9]. The chemical characteristics of the scaffold surface, such as charge, functional groups and wettability, also affect cell behaviour. The polar and positively charged surface supports the cell attachment and spreading [10], whereas the hydrophilic surface favours the adsorption of cell adhesive proteins (e.g. laminin, vitronectin, fibronectin) [6,11]. Likewise, the rough bone scaffold surface facilitates the proteins adsorption [8,10], which is critical for cell attachment, spreading, and proliferation. It is worth noting that cells do not interact directly with the biomaterial surface but with the adsorbed proteins [6].

As it was mentioned above, the cellular response to tissue-engineered constructs may depend on many factors. Thus, novel scaffolds should be subjected to a complete biological and physicochemical analysis. In this study, we fabricated the highly porous scaffold composed of the chitosan-agarose matrix reinforced with the hydroxyapatite nanopowder. The scaffold composition was to mimic bone and accelerate bone regeneration. The polysaccharide matrix was designed to imitate flexible organic parts of bone, whereas the synthetic hydroxyapatite nanopowder was to mimic the natural bone mineral [6]. Moreover, in order to obtain a highly porous structure with interconnected pores, we produced the scaffold applying a gas-foaming agent and the freeze-drying method simultaneously. Both the scaffold composition and the applied production method are characterized by high novelty and claimed in the Polish patent application no P.426788. The novel scaffold was proved to have the total open porosity (approx. 70%) and the compressive strength values (1.4 MPa) comparable to cancellous bone. Moreover, the chitosan/agarose/hydroxyapatite material is non-toxic and it favours the cell attachment and spreading [12].

Since it is known that various cell lines may exhibit different behaviours on the biomaterial surface, the primary goal of this work was to evaluate and compare the biological response of the mouse calvarial preosteoblast cell line (MC3T3-E1 Subclone 4) and the normal human foetal osteoblast cell line (hFOB 1.19) to the fabricated scaffold. Within this study, we evaluated the osteoblasts number after the 3-day culture on the biomaterial surface and the level of osteogenic markers produced by the mouse MC3T3-E1 cells and the human hFOB 1.19 ones. Additionally, changes in calcium (Ca^{2+}) and phosphorous (HPO_4^{2-}) concentrations in the culture media (dedicated for MC3T3-E1 and hFOB 1.19 cells) after incubating the scaffold were assessed. Thus we determined the ion reactivity of the material which also may influence the cellular response.

Materials and Methods

Materials

Chitosan, agarose, hydroxyapatite nanopowder (nanoHA) and sodium bicarbonate ($NaHCO_3$) were purchased from Sigma-Aldrich Chemicals. Acetic acid (CH_3COOH) and sodium hydroxide solution (NaOH) were obtained from Avantor Performance Materials. Cell experiments were performed using osteoblast cell lines: the mouse calvarial preosteoblast cell line (MC3T3-E1 Subclone 4) and the normal human foetal osteoblast cell line (hFOB 1.19), which were purchased from American Type Culture Collection (ATCC). The Alpha-MEM medium was obtained from Gibco, USA. The DMEM/Ham F12 medium without phenol red, the 0.25% trypsin-EDTA solution, the penicillin-streptomycin solution, the G418 disulfate salt solution, ascorbic acid, β-glycerophosphate, dexamethasone, paraformaldehyde, Triton X-100, bovine serum albumin (BSA) and DAPI were obtained from Sigma-Aldrich Chemicals. Lactate Dehydrogenase Activity Assay Kit (LDH) was also obtained from Sigma-Aldrich Chemicals. Foetal bovine serum (FBS) was purchased from Pan-Biotech GmbH. Ca^{2+} and HPO_4^{2-} ions concentrations in media were estimated using Calcium CPC and Phosphorous Assay Kits, which were acquired from Biomaxima. AlexaFluor635-conjugated phallotoxin was obtained from Invitrogen. Bone alkaline phosphatase (bALP) activity in cell lysates was measured using the mouse-specific ELISA assay (Mouse Bone Alkaline Phosphatase ELISA Kit) and the human-specific ELISA assay (Human Bone Alkaline Phosphatase ELISA Kit) obtained from FineTest. Type I collagen (Col I) and osteocalcin (OC) levels in cell lysates were measured using the mouse-specific ELISA assays (Mouse Collagen alpha-1(I) chain ELISA Kit, Mouse Osteocalcin ELISA Kit) and the human-specific ELISA assays (Human Collagen alpha-1(I) chain ELISA Kit, Human Osteocalcin ELISA Kit) supplied by EIAab.

Scaffold production

The scaffold was synthesized via the simultaneous application of the gas-foaming agent and the freeze-drying method. Briefly, 2%w/v chitosan (75-85% deacetylation degree, viscosity ≤ 300 cP, 50-190 kDa molecular weight) and 5%w/v agarose (low EEO, gel point 36 ± 1.5°C) were suspended in 2%v/v CH_3COOH. Then, the obtained suspension was mixed with 40%w/v nanoHA and $NaHCO_3$ (a foaming agent). The resultant paste was put into a cylinder-shaped form and subjected to heating in a water bath at 95°C. Then, the sample was cooled, frozen in a liquid vapour phase and lyophilized (LYO GT2-Basic). The final scaffold was neutralized in 1%w/v NaOH solution, washed with deionised water, and left to dry at room temperature. The scaffold was sterilized by ethylene oxide. The microstructure of the produced scaffold was visualized using a stereoscopic microscope (Olympus SZ61TR) (FIG. 1).

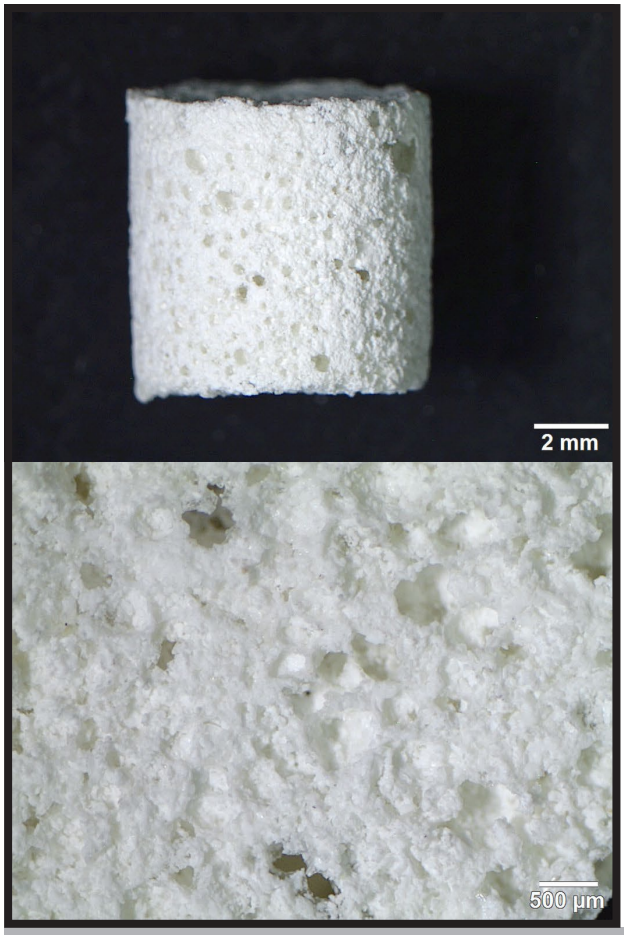

FIG. 1. Microstructure of the fabricated scaffold visualized by a stereoscopic microscope.

Ion concentrations assessment

The changes in ion concentrations were estimated in the culture media (dedicated for MC3T3-E1 and hFOB 1.19 cells) after the incubation with the scaffold. The biomaterial discs were immersed in the alpha-MEM and DMEM/Ham F12 medium maintaining the proportion of 100 mg sample per 1 ml culture medium and incubated at 37°C for 24 h. Culture media without the scaffold were treated as the control media. After 24-h incubation with the scaffold, the culture media were collected by centrifugation and Ca^{2+} and HPO_4^{2-} concentrations were estimated spectrophotometrically using the Calcium CPC and Phosphorous Assay Kits following the manufacturer's protocol.

Cell culture experiments

Prior to cell seeding onto the scaffold surface, the sample discs were placed in the wells of polystyrene plate and preincubated in the appropriate complete culture medium. The MC3T3-E1 cell line was maintained in the alpha-MEM medium supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The hFOB 1.19 cell line was maintained in the DMEM/Ham F12 medium without phenol red supplemented with 10% FBS, 300 µg/ml G418, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The MC3T3-E1 cells and the hFOB 1.19 cells were incubated in a humid atmosphere with 5% CO_2 at 37°C and 34°C, respectively.

Osteoblast number assessment

The assessment of osteoblast number on the scaffold surface was conducted after the 3-day culture. The MC3T3-E1 and hFOB 1.19 cells were seeded directly on the scaffold discs (4 mm in diameter and 2 mm thick), placed in a 96-wells plate in 100 µl of the medium at the concentration of 5 x 10^4 cells/ml. On the 3rd day, the cells grown on the surface of the scaffold were lysed, as described previously [13]. Then, the total cell number in lysates was estimated using LDH Activity Assay following the manufacturer's protocol. The exact number of cells was estimated using a calibration curve made for the known cell number of MC3T3-E1 cells and hFOB 1.19 cells.

Additionally, osteoblasts on the scaffold surface were visualized by fluorescent staining of cytoskeleton and nuclei. After the 3-day culture, the cells were fixed with 3.7%v/v paraformaldehyde, permeabilized with 0.2%v/v Triton X-100 and blocked with 1%w/v BSA. The cytoskeleton filaments and nuclei were stained with AlexaFluor635-conjugated phallotoxin and DAPI, respectively. The stained cells were visualized by confocal laser scanning microscope (CLSM).

Evaluating the level of osteogenic markers

The osteogenic differentiation of the cells on the scaffold surface was carried out for 16 days. The MC3T3-E1 cells and the hFOB 1.19 cells were seeded directly on the scaffold discs (7 mm in diameter and 2 mm thick), placed in a 48-wells plate in 500 µl of the appropriate complete culture medium at the concentration of 4 x 10^5 cells/ml and incubated for 24 h at 37°C and 34°C, respectively. Then, the culture medium was discarded and replaced with the osteogenic medium made of the appropriate complete culture medium supplemented with 50 µg/ml ascorbic acid, 0.01 µM β-glycerophosphate, and 0.01 µM dexamethasone. Every third day, half of the osteogenic medium was changed with a fresh portion. On the 8th and 16th day of the experiment, the cells were lysed, as described previously [13], and the level of osteogenic markers in cell lysates was estimated using appropriate ELISA assays for mouse and human species. The bALP activity and Col I concentration were estimated on the 8th day of the experiment, whereas the bALP activity and OC concentration were estimated on the 16th day of the experiment.

Statistical analysis

All the experiments were conducted in triplicate (n = 3) and the obtained results were showed as mean values ± standard deviation (SD). The data were statistically analyzed using an unpaired t-test (GraphPad Prism 8.0.0 Software). Statistically significant differences were considered at $p < 0.05$.

Results and Discussions

Ion concentrations assessment

The surface of calcium phosphate-based biomaterials may interact with the ions which are present in the culture environment, causing fluctuation in ion concentrations which, in turn, may influence cellular response [14,15]. Thus, in this study, the evaluation of ion concentrations in the culture media after the scaffold incubation was performed. In this experiment, the media dedicated particularly for maintaining the MC3T3-E1 and the hFOB 1.19 cell were used. As shown in FIG. 2a, the biomaterial incubation in the culture medium dedicated for the MC3T3-E1 cell line resulted in the significant uptake of Ca^{2+} ions from the surrounding microenvironment. Interestingly, the divergent results were obtained for the scaffold incubated in the culture medium dedicated for the hFOB 1.19 cell line (FIG. 2b) where the significant release of Ca^{2+} ions was observed. As for the HPO_4^{2-} concentration in the culture media, it was revealed that the scaffold caused the significant uptake of these ions, regardless of the applied culture medium. The observed fluctuations in ion concentrations in the liquid environment are typical for calcium phosphate-based biomaterials. The uptake of Ca^{2+} and HPO_4^{2-} ions from the culture medium probably resulted from the electrostatic interaction between the charged biomaterial surface and ions in the culture microenvironment [16]. Moreover, this phenomenon may be also associated with the bone-like apatite formation on the scaffold surface [16,17]. The Ca^{2+} ions release was possibly caused by the hydroxyapatite dissolution or the ionic substitution of Ca^{2+} ions present in hydroxyapatite by other ions occurring in the culture medium [18].

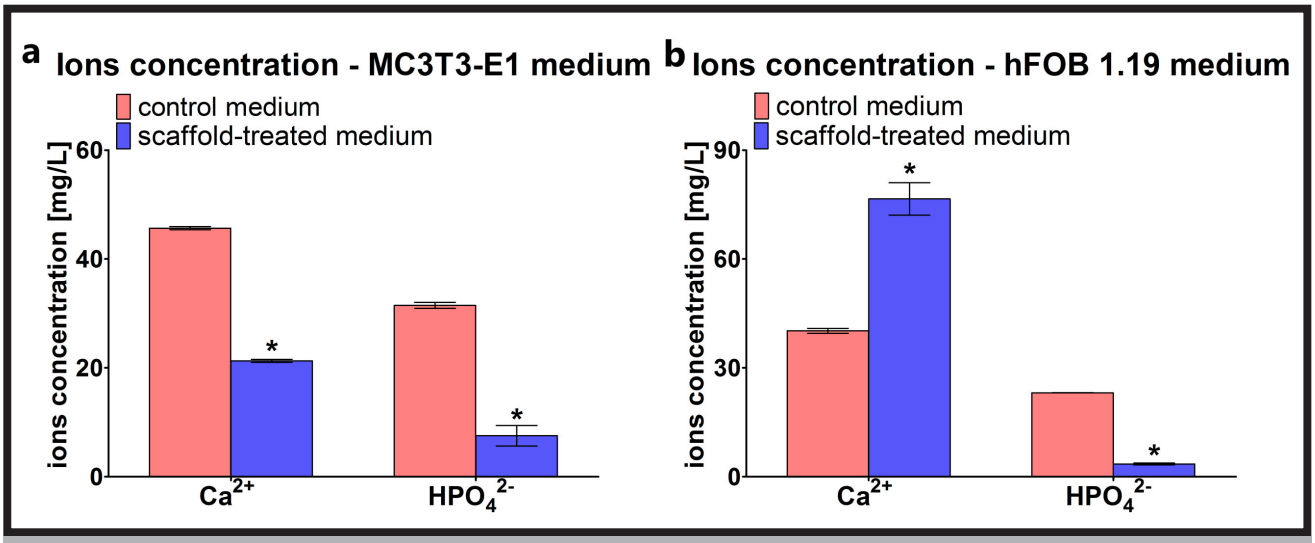

FIG. 2. Changes in Ca^{2+} and HPO_4^{2-} ions concentration [mg/L] in culture medium dedicated for (a) MC3T3-E1 and (b) hFOB 1.19 cells after incubation with the scaffold (scaffold-treated medium); *statistically significant results compared to appropriate control medium ($p < 0.05$, n = 4, unpaired t-test).

Osteoblast number assessment

To compare cell behaviour of the two different osteoblast cell lines on the scaffold surface, the mouse (MC3T3-E1) and human (hFOB 1.19) osteoblasts were seeded directly on the scaffold and cultured for 3 days, then the cell numbers were evaluated by the LDH total test. FIG. 3 shows that after the 3-day culture, the number of the MC3T3-E1 cells was 8.99 ± 1.04 x 10^3, whereas the number of the hFOB 1.19 cells equalled 7.14 ± 1.65 x 10^3. Thus, the MC3T3-E1 cells were slightly more numerous on the material surface as compared to the hFOB 1.19 osteoblasts. However, the observed differences were not statistically significant. It should be noted that the cell growth on the scaffold surface may be affected by fluctuations in ion concentrations occurring in the surrounding culture medium. It is well known that extracellular Ca^{2+} ions support osteoblast adhesion, proliferation and extracellular matrix (ECM) formation [8]. However, too high Ca^{2+} concentrations in the culture microenvironment may lead to hyperosmotic stress causing cell shrinkage and intracellular dehydration, followed by cell death [19]. It was observed that the scaffold caused the increase in Ca^{2+} concentration in the culture medium dedicated for human osteoblasts. Thus, a slightly lower number of the hFOB 1.19 cells on the scaffold surface as compared to the mouse osteoblasts could result from local too high concentration of Ca^{2+} ions.

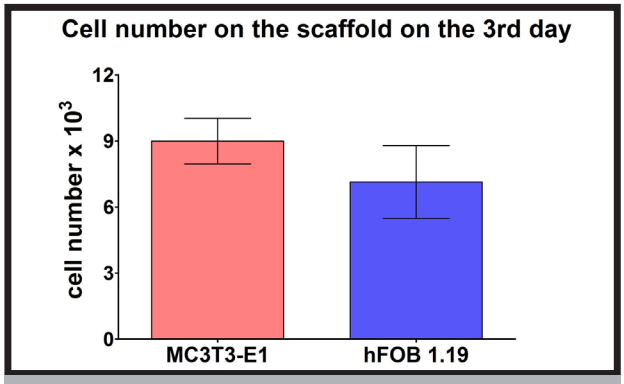

FIG. 3. Comparison of osteoblast number on the surface of the scaffold (n = 4, unpaired t-test).

The CLSM images showed that both the MC3T3-E1 cells and the hFOB 1.19 cells cultured on the scaffold surface were well spread and had flattened morphology (FIG. 4), proving that the surface of the fabricated scaffold supported the cell attachment and growth. Thus, the developed biomaterial possesses osteoconductive properties which are defined as the ability of the scaffold to favour cell adhesion, growth, and differentiation [6].

Evaluation of osteogenic markers level

The osteogenic differentiation of cells is a 3-step process involving: 1) intensive cell proliferation, 2) ECM synthesis, 3) ECM mineralization. During each phase of the osteogenic differentiation, cells produce specific osteogenic markers. During the proliferation stage, cells exhibit rapid proliferation and produce mainly a great amount of Col I and fibronectin. In the ECM synthesis stage, cells do not divide anymore and begin the intensive synthesis of the bone ECM proteins. Additionally, during this stage cells exhibit the highest bALP activity. In the third stage, cells reveal high mineralization activity. This phase is also characterized by high production of OC and osteopontin which are responsible for binding calcium ions and thus ECM mineralization. Importantly, during the ECM mineralization stage, the bALP activity is at a moderate level [6,20].

The evaluation of osteogenic cells differentiation on the surface of the engineered bone scaffold is crucial in terms of medical application. In this study, we tested *in vitro* two cellular models (MC3T3-E1 Subclone 4 and hFOB 1.19) which are commonly used in preclinical testing of biomaterials and are considered proper models for studying osteoblast behaviour and osteogenic differentiation [6]. The MC3T3-E1 cell line is known for cell proliferation and mineralization potential similar to human primary osteoblasts [21]. Moreover, MC3T3-E1 Subclone 4 forms a well mineralized ECM and exhibits high mRNAs expression for osteogenic markers, such as OC, parathyroid hormone (PTH)/parathyroid hormone-related protein (PTHrP) receptor and bone sialoprotein while cultured in presence of ascorbic acid and inorganic phosphate [22]. Whereas, the hFOB 1.19 cell line is considered a great cellular model, since it may differentiate into mature osteoblasts with the phenotype similar to normal primary osteoblasts [23].

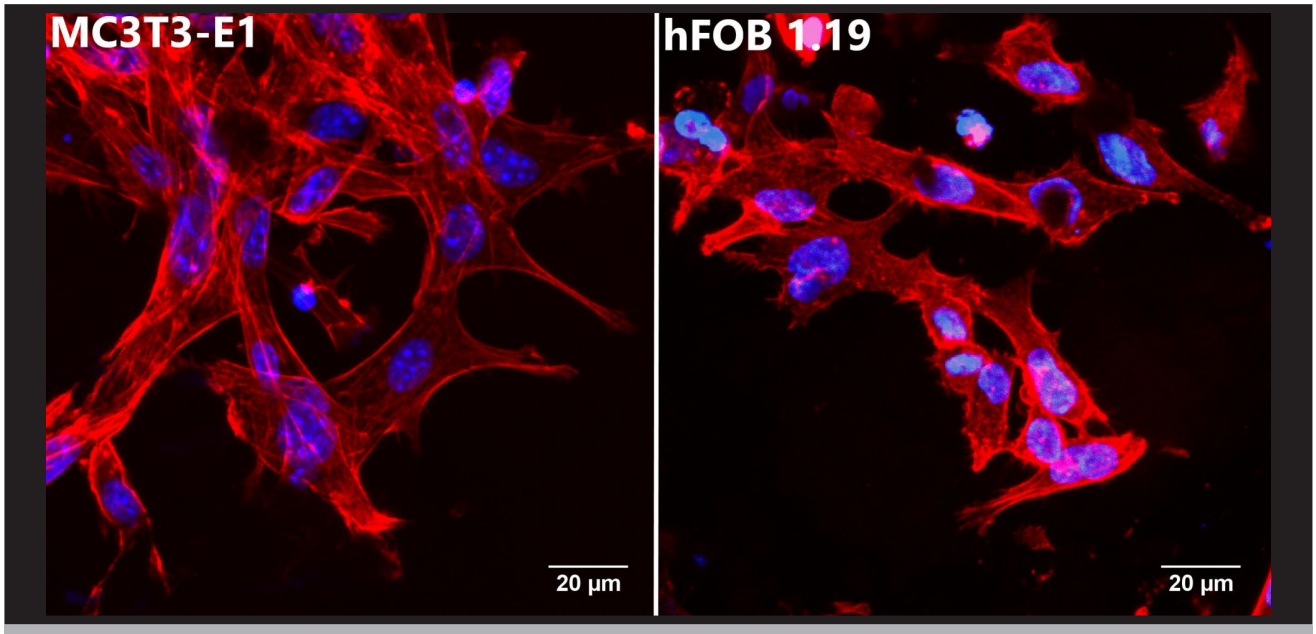

FIG. 4. Visualization of osteoblasts grown on the scaffold surface (cytoskeleton filaments – red fluorescence, nuclei – blue fluorescence; magn. 400x, scale bar = 20 µm).

In this research, the level of typical osteogenic markers (bALP, Col I, OC) produced by the MC3T3-E1 and the hFOB 1.19 cells cultured on the scaffold surface was estimated. The bALP activity was evaluated on the 8th and 16th day of the experiment since this enzyme is especially important for the second and the third stage of the differentiation process. Col I which is characteristic of the first and second stage was determined only on the 8th day, whereas OC, which is a late marker, was determined only on the 16th day of the experiment. The performed ELISAs clearly showed that the level of osteogenic markers which were synthesized by the cells cultured on the scaffold surface depended on the cell line type (FIG. 5).

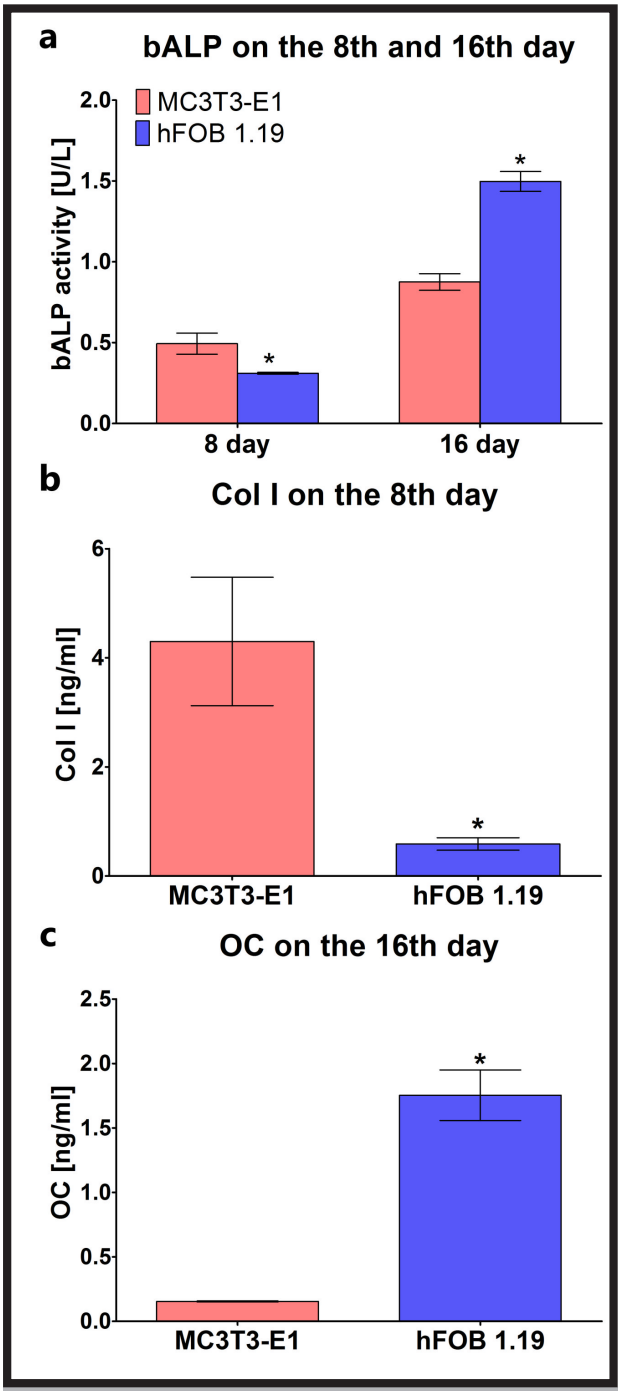

FIG. 5. Evaluation of osteogenic markers level: (a) bALP, (b) Col I, and (c) OC in MC3T3-E1 and hFOB 1.19 osteoblasts cultured on the surface of the scaffold for 8 and 16-days; *statistically significant results compared to MC3T3-E1 cell line ($p < 0.05$, n = 4, unpaired t-test).

It was observed that on the 16th day, the bALP activity increased when compared to the 8th day of the experiment for both cell lines (FIG. 5a). However, on the 16th day of the experiment, the hFOB 1.19 cells exhibited a significantly higher bALP activity than the MC3T3-E1 cells. Interestingly, the MC3T3-E1 cells produced a significantly higher amount of Col I than the hFOB 1.19 cells (FIG. 5b), whereas the hFOB 1.19 cells produced a significantly higher amount of OC than the MC3T3-E1 cells (FIG. 5c). Since human osteoblasts revealed the higher bALP activity (typical of 2 and 3 phase), they produced lower amounts of Col I (early differentiation marker, typical of 1 and 2 phase) and synthesized greater amounts of OC (late marker, typical of 3 phase) in comparison to mouse cells, it may be concluded that the hFOB 1.19 cells were in a more advanced phase of osteogenic differentiation than the MC3T3-E1 cells. The lower differentiation degree of the mouse cells cultured on the scaffold most likely resulted from the preosteoblast phenotype of MC3T3-E1 cells, as compared to human osteoblasts. According to the available literature, in contrast to the MC3T3-E1 cells, the differentiated hFOB 1.19 cells exhibit phenotype and gene expression typical of primary mature osteoblasts [6]. Importantly, the obtained results clearly demonstrated that the surface of the fabricated scaffold facilitated the osteogenic differentiation, confirming that the developed biomaterial has osteoconductive properties.

Conclusions

The obtained results demonstrated that the fabricated scaffold composed of the chitosan-agarose matrix reinforced with hydroxyapatite nanopowder is characterized by biocompatibility and osteoconductivity. The scaffold allows for the attachment and growth of both the mouse calvarial preosteoblasts (MC3T3-E1 Subclone 4) and the normal human foetal osteoblasts (hFOB 1.19). The innovative biomaterial also promotes the production of osteogenic markers by the mentioned cells. Interestingly, the developed scaffold reveals the different ion reactivity in the culture medium dedicated for mouse cells in comparison to the medium for human osteoblasts. It was also proved that the cell lines may differ in the cellular response to the investigated biomaterial. Therefore, to yield more reliable results it is recommended to perform biological characterization of the novel scaffold using at least two various cellular models. The presented results indicate that the novel bone scaffold has a great potential to be used in bone regeneration applications, however further experiments need to be performed to fully confirm its biomedical usefulness.

Acknowledgments

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