Calcium phosphate composite cements based on simple mixture of brushite and apatite phases

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Abstract. The composite cements based on simple mixtures brishite and apatite with ratio 70/30, 50/50, 30/70 were developed. The processes of phase formation, microstructure and mechanical properties were studied. The kinetics of degradation in simulated body fluid depending on the microstructure and the materials phase composition was carried out. The biological test *in vitro* were performed using the MTT-test on the human fibroblast immortalized (hFB) cell line and the human osteosarcoma cell line MG-63. The materials didn't have acute cytoxicity and possessed surface matrix properties. It was determined that the both line of cells actively proliferated, with viable cells values higher 20-60 % then control at all observation periods.

1. Introduction
Nowadays, the replacement of bone tissue is regarded as one of the most common surgical procedures. In fact a variety of techniques and synthetic substitutes have been developed in order to properly reconstruct tissue defects. CPCs represent a adequate synthetic substitute, which are widely used as filling materials in the field of orthopedic and dental medicine because of their good biocompatiblity, resorbability and a low toxicity [1]. The type of setting product formed by the reaction is affected by the pH value conditions in the cement paste. The two most commonly encountered end products are precipitated apatite or brushite. Brushite cements possess resorption rate quicker than apatite cements due to the higher solubility of brushite phase compared to that of calcium-deficient hydroxyapatite, which may be an advantage for bone regeneration [2].

Despite numerous advantages, it is widely accepted that CPCs need further improvements to their properties to broaden their potential clinical applications. The properties of CPCs are affected by many factors. Therefore, it is imaginable that all the factors, such as the chemical composition of the cement, the relative proportions of the reactants in the mixture, powder or liquid additives acting as accelerators or retarders, particle size, liquid / powder ratio, pressure applied during sample preparation and aging conditions, will affect its properties [3]. Many of the studies directed to improving CPC properties for clinical applications. Most of them were focused on improving next points: processing techniques [5], CPC mechanical properties [6], influence of CPC material properties on cell behavior [7], CPC materials as drug delivery systems [8-9], stem cell delivery by CPC [9]. The improvement of these properties is carried out by introducing a different polymer, fiber or ceramic additives in CPC based on brushite or

apatite. Therefore, when optimizing any property of CPC, it is important to consider all the crucial properties. The main idea of this work, the development of composite materials by combining the advantage brushite and apatite cements. The study of these cements are poorly described in the literature.

On the other hand, the disadvantage of these materials is low porosity, do not contribute to the integration of the cement with the surrounding bone tissue. The absence of interconnected pores disturbs the circulation of the liquid inside the cement and the absence of fine pores reduces the adhesion of cells on the surface of the cement. The creation of porous cements with different pore-forming additives (PA) can solve this problem. PA introduced at the stage of mixing the, for example, crystals of soluble salts, particles of sugar, ice, water-soluble polymers cement and removed after hardening of the binding system. With the introduction of such additives can be achieved with a porosity of 50 %. Also, porosity in cements can be created and with the use of foaming agents, for example, ammonium carbonate or carbonate of calcium, the interaction of which with the cement liquid (usually phosphoric acid) is accompanied by release of carbon dioxide. It is possible to achieve higher porosity, but it is difficult to control the pore size [10, 11].

The purpose of this study was to prepare brushite/apatite composite cements and investigate the effect of PA on the phase formation, microstructure and mechanical properties, *in vitro* cytocompatibility and matrix (for cells) properties in dynamics of cultivation (up to 7 days) of the final cement composites.

2. Experimental

In this work the composition of CPCs were chosen: brushite cement (dicalcium phosphate dihydrate (DCPD)) based on reaction of the metastable hydrolysis of orthophosphate in the aquatic environment and apatite cement (apatite (HA) products) based on acid reaction with the formation of a neutral compound.

The cement paste was prepared by manually mixing the powder α-tricalcium phosphate (α - TCP) average size of 1-10 μm with hardening liquid (HL) comprising a magnesium phosphate solution in orthophosphoric acid with a spatula in a glass mortar, maintaining a liquid-to-powder ratio for brushite cement of 0.25 ml g^{-1} and apatite cement of 0.22 ml g^{-1}. Composite cement prepared by simple mechanical mixture apatite and brushite cement paste with 70 wt.%/ 30 wt.%, 50 wt.% / 50 wt.%, 30 wt.% / 70 wt.%.

Porous CPCs were prepared by the standard procedures reported in literature [12] using two types of PA: chemical and soluble additives. The introduction of chemical-reactive PA ($CaCO_3$, Na_2CO_3) to the CPC leads to chemical interaction with CPC. It was resulted from obtaining and releasing of CO_2 gas. The formation of the tunnel interconnected pores with a size about 30-40 μm and porosity up to 30 % was occurred. The porosity formation soluble PA (NaCl) was due to dissolution PA with contact simulated body fluid. PA were taken in a quantity of up to 5 wt.% for Na_2CO_3 and 15 wt. % for $CaCO_3$ and NaCl.

The cement setting time was determined with a Vicat apparatus based on the instant of disappearance of the Vicat needle trace on the sample surface. The resulting mass was put into 6 mm diameter × 12 mm height cylindrical Teflon moulds. After 5 – 7 minutes, the sample were allowed to set at 100 % relative humidity for 7 days at 37 °C.

The samples were investigated by scanning electron microscopic analysis (SEM, Tescan Vega II). The secondary and backscattered electron modes was used for microstructure studies of the ceramic samples. The samples were coated with a 25 nm-thick gold layer to impart electrical conductivity to the specimen surfaces for SEM.

The phase composition was analyzed by conventional X-ray diffraction (XRD) technique (Shimadzu XRD-6000 (Japan), Ni-filtered $CuK_{\alpha 1}$ target, λ=1.54183 Å).

The porosity, intergranular size and specific surface area of the investigated materials were determined by mercury porometry (TriStar 3000, Micromeritics, USA).

The mechanical tests were carry out by axial compression of cylindrical samples using Instron 5581 testing machine. The solubility testing was carried out according to GOST R ISO 10993-14-2001 by

measuring of mass loss. The samples were kept in buffering solution up to 28 days at a constant volume of the liquid phase (closed system), pH 4.5, and 37°C.

The kinetics of degradation was evaluated by weight loss CPCs when exposed in SBF up to 28 days. The formation of calcium phosphate layer was investigated by scanning electron microscopy. In this study we used the SBF consist of composition (g/L): NaCl 6.457; NaHCO$_3$ 2.268; KCl 0.373; Na$_2$HPO$_4$ · 12H$_2$O 0.178; MgCl$_2$ · 6H$_2$O 0.305; Na$_2$SO$_4$ 0.071; CaCl$_2$ ·2H$_2$O 0.368; Tris buffer 6.057.

The biological studies of CPCs in vitro were assessed on the hFB, clone 1608 hTERT (Engelhardt Institute of Molecular Biology of Russian Academy of Sciences) and the human osteosarcoma cell line MG-63 (Russian Cell Culture Collection, Institute of Cytology of The Russian Academy of Sciences). Before in vitro experiments, sterile CPC samples (γ irradiation of 15 kGy) were placed into 96 well plates for cultivation (Costar, United States) in triplicate and one plate per each incubation period (1, 3,7 days) and covered with complete growth medium (CGM) that contained DMEM medium (PanEko,Russia), 10% fetal calf serum (FCS, Austria), glutamine (0.65 mg/mL, PanEko,Russia), and gentamycin (50 μg/mL, PanEko,Russia). After neutral values of pH were established (1–3 days of the sample exposure to the CGM with a change of medium), the cell suspension (hFB at density of 2x104 cells per well or MG-63 at 1,5x104 cells per well) at 200 μL CGM was added to the plates with samples (experiment) and without them (control with cultural plastic, polystyrene). All the procedures were performed under sterile conditions in an atmosphere of moist air that contained 5% CO2 at 37°C. The viability of the hFB and MG-63 cell lines over time was assessed using an MTT test [13]. Briefly, after appropriate co- culture incubations, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma–Aldrich, USA,5 mg/mL) was added to each well and incubated for 3 h until coloured formazan compounds were visible. The supernatants were aspirated and isopropanol was added to dissolve formasan precipitates. Optical density at 540 nm was measured using an automatic reader for microtiter plates (Multiscan FC, Thermoscientific USA).

At cultivation stages, a pool viable cell (PVC) was calculated as a ratio of the optical density of the formazan solution in the experiment to this value in the control. The results were processed by conventional methods of variational statistics using Microsoft Excel 2000. The significance of differences was assessed using a parametric Student t-test; differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

First, the most promising formulation was selected to investigate the phase formation after the setting reaction. For this purpose, the effect of the brushite / apatite cement ratio in the mixture was investigated in order to find the reasonable values for phase composition, setting time and compressive strength. Table 1 gives the results obtained for these parameters.

Table 1 Characteristics of CPC cements as a function of composition.

Composition phase mixture (wt. %)	Final composition phase (wt%)	Setting time (min)	Compressive strength (MPa)
brushite	DCPD/HA/TCP 85/10/5	8	6
brushite/apatite 70/30	DCPD/HA/TCP 75/20/5	9	22
brushite/apatite 50/50	DCPD/HA/TCP 65/30/5	10	28
brushite/apatite 30/70	DCPD/HA/TCP 10/80/10	13	35
apatite	HA/TCP 90/10	15	55

It was shown that very small amount of the hydroxyapatite (HA) phase (10 wt. %) was detected for brushite cement, whereas about 20 wt. % of HA in the final reaction product was obtained for the 70/30 brushite/apatite composition. The (65 and 10 wt. %) DCPD, (30 and 80 wt. %) HA phases were detected for 50/50 and 30/70 brushite/apatite compositions. The setting time increases with increasing content of apatite phase. This the dependence of phase formation can be justified by a difference in solubility

between DCPD and HA increasing upon the HA conversion. Recently, we reported the additional component (DCPD) influences significantly the structural behavior of HA, acting as the HA crystallization inhibition agent [14].

Further it was chosen the materials with the following composition: brushite, 50/50 and apatite. This choice of materials was made due to the following reasons: brushite/apatite 50/50 composition had optimal ratio of setting time and compressive strength. Brushite and apatite were chosen as extreme concentrations.

The XRD analysis had shown that apatite cements had an amorphous structure, with α-TCP particles up to 40 wt. %. The composite cements 50/50 had amorphous apatite phase (up to 30 wt. %), brushite phase (up to 30 wt. %) and crystalline α-TCP (up to 40 wt. %). The brushite cements had amorphous apatite phase (10 wt. %), brushite phase (up to 70 wt. %) and crystalline α-TCP (up to 20 wt. %). The addition of $CaCO_3$ in an amount of 5 wt. % brushite cement leads to the formation of apatite phase up to 10 wt. %. The introduction of additives in apatite cement had no effect on the phase composition (Figure 1).
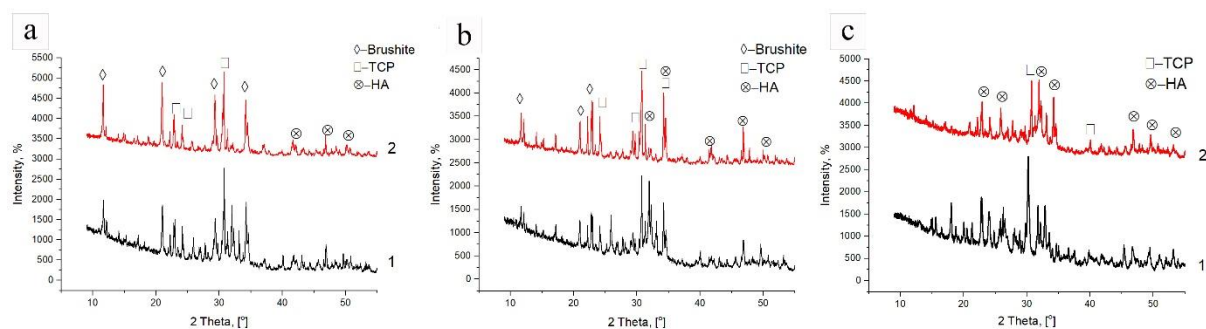
**Figure 1**. Comparative XRD pattern (1) dense and (2) porous CPC: (a) brushite; (b) 50/50, (c) apatite.

According to SEM observation the CPCs cement was predominantly homogeneous with α-TCP particles with average size of 1-10 µm embedded into the matrix (Figure 2). Brushite and 50/50 cements are composed of plate shape brushite crystals up to 2 µm thick and 5-20 µm length, with α-TCP particles and apatite phases.
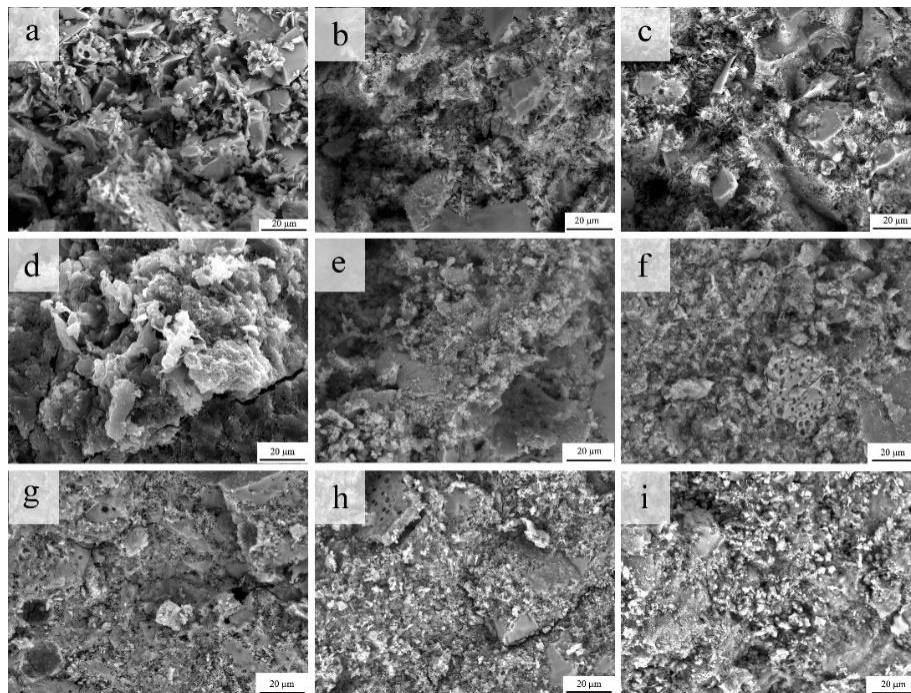
**Figure 2**. SEM images of the porous CPC samples with PA wt. 5 %: (a) – brushite with $CaCO_3$, (b) - brushite with Na_2CO_3, (c) - brushite with NaCl, (d) – 50/50 with $CaCO_3$, (e) – 50/50 with Na_2CO_3, (f) – 50/50 with NaCl, (g) – apatite with $CaCO_3$, (h) - apatite with Na_2CO_3, (i) - apatite with NaCl.

As shown in Figure 3, the interaction between brushite and apatite cement didn't occur. Uniform distribution of phases was observed.
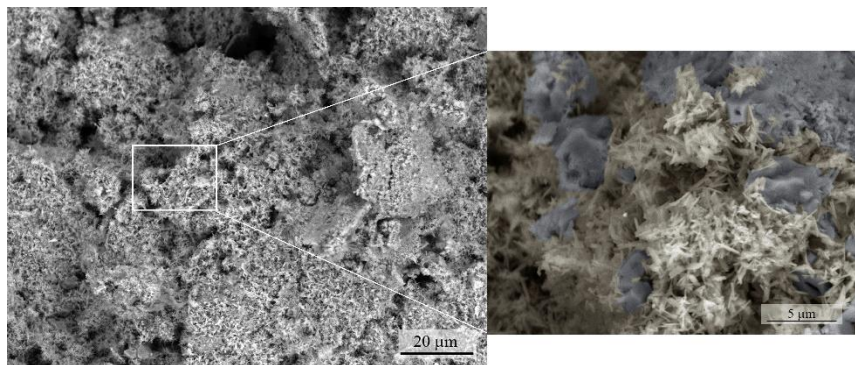

Figure. 3. Microstructure of the 50/50 composite material.

The open porosity dependence on PA (Figure 4) is linear and increases till the PA maximum concentration up to 15 wt. %. The samples obtained with the injection of the $CaCO_3$ and Na_2CO_3 have a high level of open porosity to 25 and 22 %, respectively, due to the release of CO_2 gas when the additive interacts with the hardening liquid. Such materials have interconnected pores of irregular shape with a size of 1-20 µm; wherein the gas realise posed by the initial reagents reaction had form of the tunnel porosity. The injection of NaCl leads to the formation of spherical pores with of 1 - 5 µm a size.
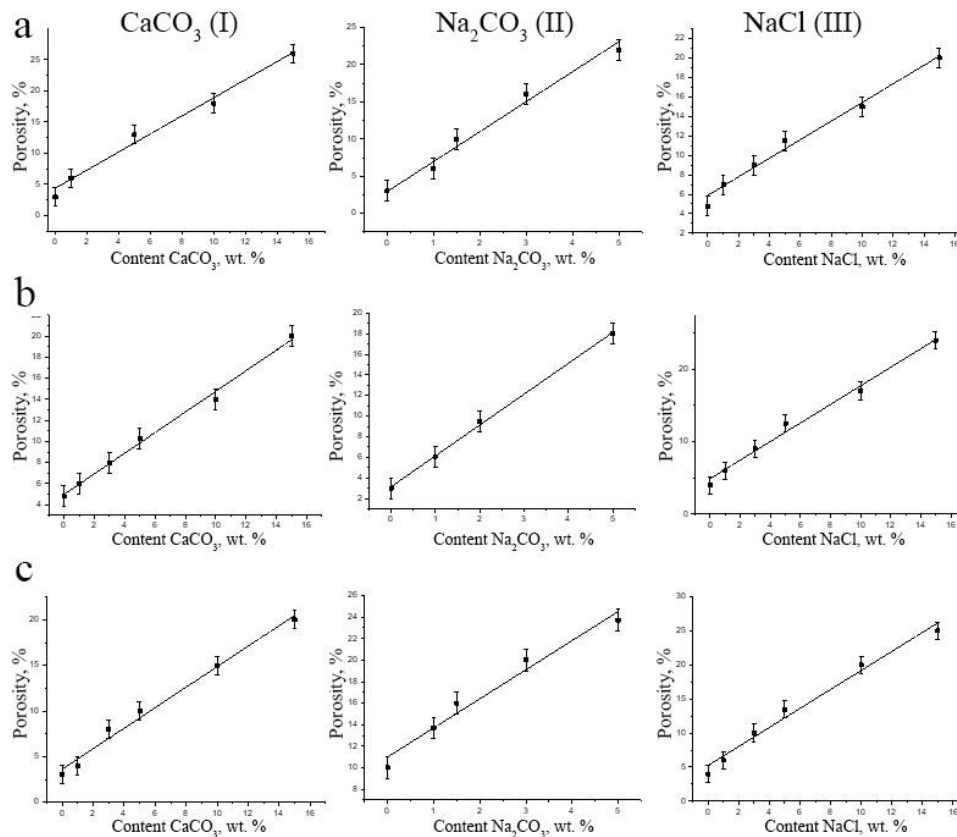
Figure 4. Dependence of porosity on the content and type of the additive: (a) – brushite, (b) - 50/50, (c) – apatite, (I) – CaCO₃, (II) – Na₂CO₃, (III) – NaCl.

The compressive strength of the CPCs increased from $6 \pm 0,5$ MPa for brushite, to 25 ± 3 MPa brushite/apatite (50/50) and 50 ± 3 MPa for apatite (Figure 5). It is established that the compressive strength values of CPCs monotonically decrease when increasing amount of PA, which is consistent with the results of open porosity. The highest compressive strength are for samples with $CaCO_3$ with 15 wt. %: 4, 16 and 27 MPa, respectively, for brushite, 50/50 and apatite cements. Based on the results CPC 50/50 with the additive $CaCO_3$ 5 wt. % has been chosen optimal combination of properties.
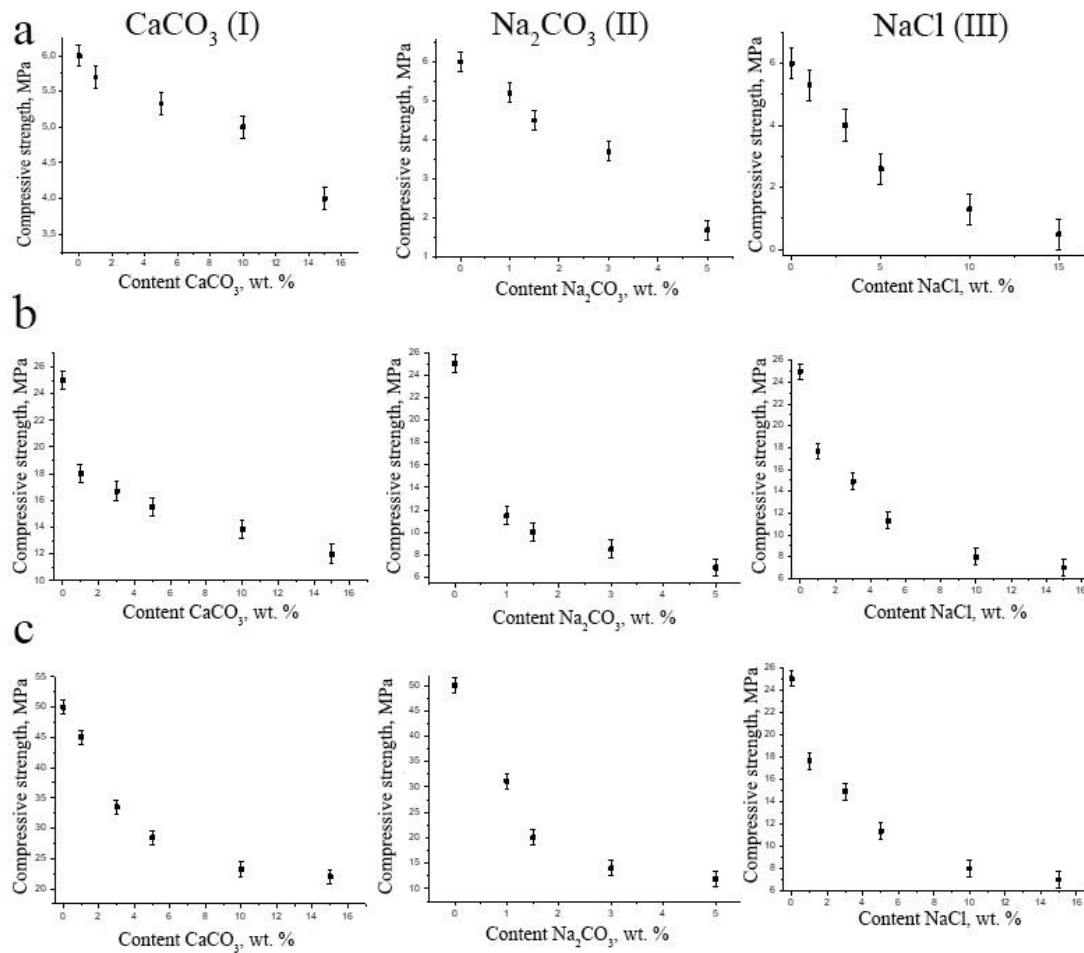
Figure 5. Dependence of compressive strength on the content and type of the additive:
(a) – brushite, (b) - 50/50, (c) – apatite, (I) – CaCO$_3$, (II) – Na$_2$CO$_3$, (III) – NaCl.

The kinetics of dissolution of CPCs of all compositions at the initial stage can be described by an exponential function (Figure 6). The mass loss of CPCs was increased significantly by 5 to 7 days in a buffer solution, and after what dissolution rate slows down. The quantitative weight loss values of CPCs were different according to the composition and porosity of the material. Weight loss for CPC 50/50 in the buffer solution were 30 - 32 % for CaCO$_3$, 15-16 % for Na$_2$CO$_3$ and 10 -12 % for NaCl. For Apatite CPC weight loss were 16-17 % for CaCO$_3$, 20-21 % for Na$_2$CO$_3$ and 23 – 24 % for NaCl.
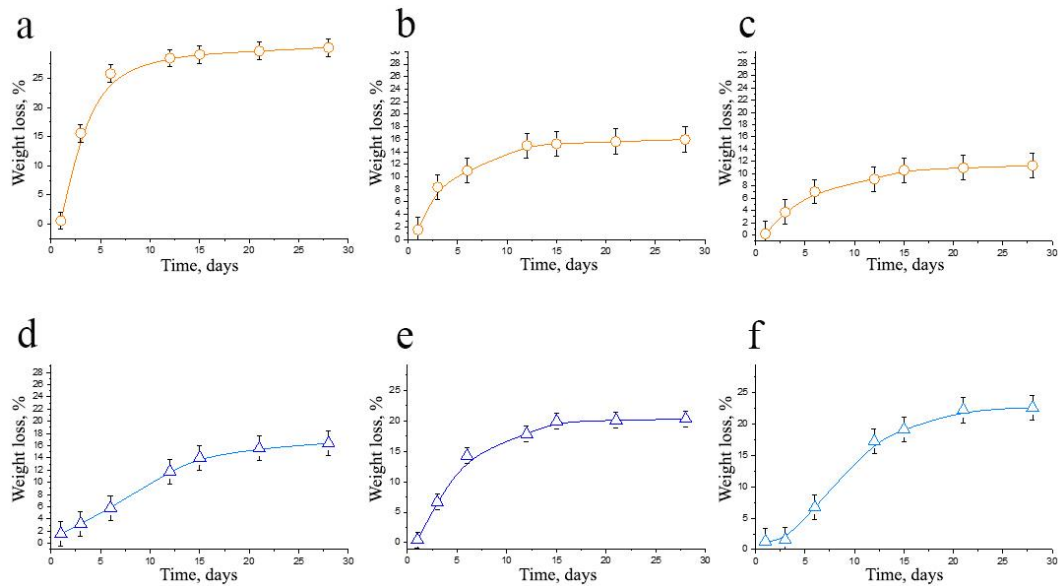
Figure 6. Kinetic of weight loss CPC in buffer solution: (a) 50/50 with CaCO$_3$, (b) – 50/50 with Na$_2$CO$_3$, (c) – 50/50 with NaCl, (d) – apatite with CaCO$_3$, (e) - apatite with Na$_2$CO$_3$, (f) - apatite with NaCl.

In vitro test of hFB and MG-63 on the CPC with 5 wt. % CaCO$_3$ demonstrated the absence of toxicity and the presence of surface matrix properties with varying degree (Figure 7). Thus, the results of the experiments indicate that the material with compositions brushite, 50/50, apatite were cytocompatibility with respect to the line of hFB and MG-63 cells. The both line cells after plating and adhesion/spreading to the CPC were actively proliferating and had viable cells values higher 20-60 % then control at all observation periods (Figure 8).
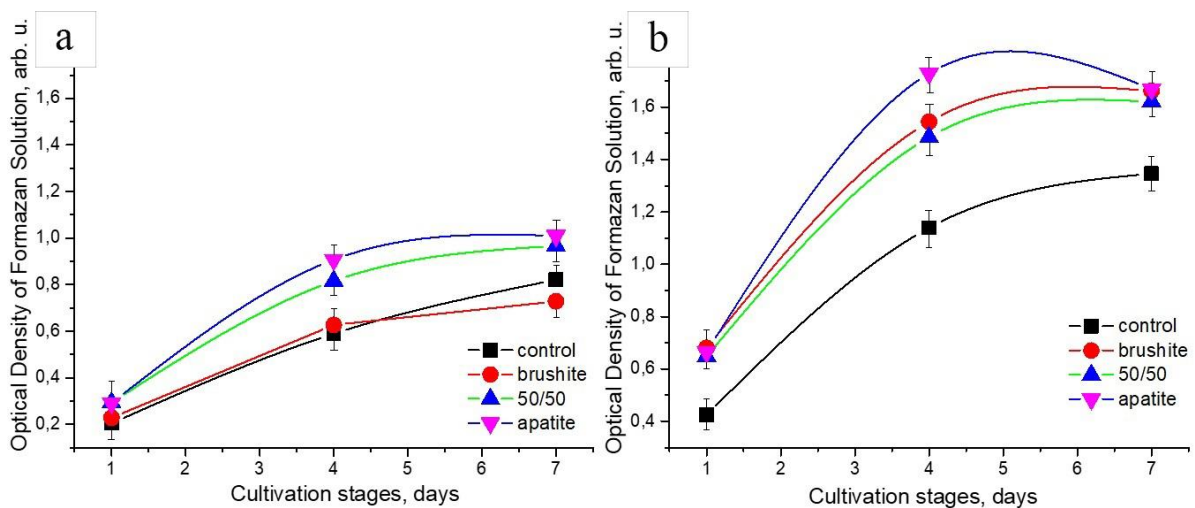
Figure 7. Increase in the population of (a) hFB and (b) MG-63 cells at cultivation stages.
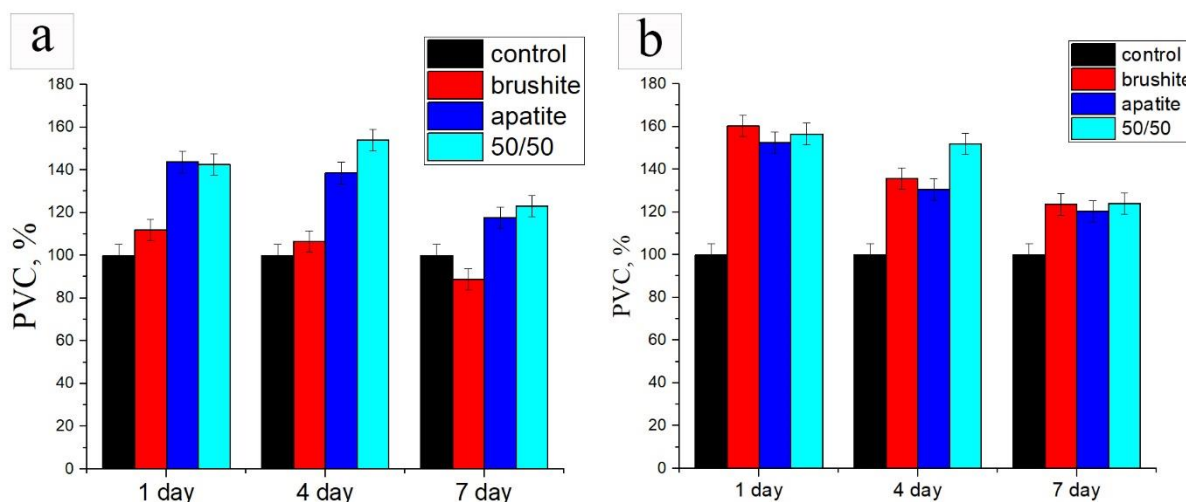
Figure 8. Value of VPC of (a) hFB and (b) MG-63 cells at cultivation stages.

4. Conclusion

The composite cements in the brushite-apatite system with different ratios were developed. The most promising composition was brushite/apatite 50/50 with the final phases DCPD/HA/TCP 65/30/5 wt. %. The pore-forming additives ($CaCO_3$, Na_2CO_3, NaCl) CPC were able to obtain a porosity from 10 to 27 %. The optimal properties CPC: setting time 10 min, porosity 15 %, compressive strength 18 MPa and weight loss 30-32% were obtained with $CaCO_3$ 5 wt. %. The absence of toxicity and the presence of appropriate surface matrix properties were demonstrated. The developed composite cements can be used for bone defects replacement.

Acknowledgments

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