

PREPARATION AND CHARACTERIZATION OF BRUSHITE CEMENTS BASED ON AMORPHOUS CALCIUM PHOSPHATE AND CARBOXYLIC ACIDS

Radost Ilieva¹, Rumiana Gergulova¹, Stefka Tepavitcharova¹, Kostadinka Sezanova¹,
Anton A. Apostolov², Boyka Andonova-Lilova³, Radostina Alexandrova³

¹Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences,
Acad. G. Bontchev Str., Bl.11, 1113 Sofia, Bulgaria

²Faculty of Chemistry and Pharmacy, Laboratory of Polymers, Sofia University,
1, J. Bourchier Blvd., 1164 Sofia, Bulgaria

³Institute of Experimental Morphology, Pathology and Anthropology with Museum,
Bulgarian Academy of Sciences, Acad. G. Bontchev Str., Bl. 25, 1113 Sofia, Bulgaria

Abstract

Three series of calcium phosphate brushite cements based on double doped amorphous calcium phosphate ((Mg,Zn)-ACP) and carboxylic acid (citric, tartaric or lactic) were prepared. The effect of the acid used, as well as that of the solid to liquid ratio on the transformation of (Mg,Zn)-ACP to brushite phase and on the forming characteristics and setting times were studied. Two types of *in vitro* tests of the cement molds were performed – long-term behavior of the molds in simulated body fluid (SBFc) and cell test with three types of cell cultures. The cement mold based on (Mg,Zn)-ACP and tartaric acid was found to display the best forming characteristics, the highest stability and the lowest cytotoxicity.

Key words: brushite cements, amorphous calcium phosphate, carboxylic acids, cell viability

1. INTRODUCTION

Calcium phosphate cements (CPCs) are developed as moldable or injectable pastes to fill bone cavities, defects or discontinuities of any geometric shape. They set and harden shortly after implantation, forming biocompatible and osteoconductive scaffolds (Gama *et al.* 2009). CPCs are generally prepared by mixing amorphous and/or crystalline calcium orthophosphate powders with an aqueous solution which might be distilled water, aqueous solution of different salts, orthophosphoric acid, etc. (Dorozhkin 2011). The setting reactions are a result of the dissolution/crystallization or hydrolysis processes taking place, which yield less soluble calcium orthophosphate compounds. Thus, according to the final phase, two types of CPCs are known: apatite and brushite cements (Bohner *et al.* 2005, Rau *et al.* 2010). Although hydroxyapatite is the calcium phosphate compound of lowest solubility and the thermodynamically most stable phase at pH values higher than 4.2, the final phase of apatite cements is poorly crystalline non-stoichiometric calcium deficient hydroxyapatite that forms in real cement formulations at pH values higher than 6 (Dorozhkin 2011). Below pH 6, the final phase is brushite (dicalcium phosphate dihydrate, DCPD). The brushite cements have raised special interest because they are resorbed *in vivo* much faster than apatite ones (Schneider *et al.* 2010), Theiss *et al.* 2005, Bohner & Gbureck 2008) due to brushite metastability under physiological conditions. Moreover, brushite based cements possess shorter setting times (Dorozhkin 2011) than apatite ones. The major disadvantage of brushite cements is their low mechanical strength.

During the past two decades many studies have been aimed at improving the properties (setting time, mechanical properties, biocompatibility, bioactivity and bioresorption) of brushite cements by varying the type and ratio between the initial solid and liquid phases and also using different additives. Since brushite cements are set by acid-base interaction, usually basic calcium phosphate salts as calcium deficient hydroxyapatite (Lilley *et al.* 2005), tricalcium phosphate (α or β) (Pina *et al.* 2010, Kannan *et al.* 2006, Van Landuyt *et al.* 1997) or tetracalcium phosphate (Sawamura *et al.* 2016) in different combinations are used as the solid phase. Phosphoric acid (Lilley *et al.* 2005), citric acid (Engstrand *et al.* 2014), glycolic acid (Marino *et al.* 2007), etc., are used as the liquid phase. The presence of various

ions other than calcium and phosphate ones in the cement has important effects on the reaction taking place and on the final properties of the material. Metal ions can be added to the cement simply by mixing the cement powder phase with a salt containing the ion or by doping the cement precursors with different ions. In this way, new brushite cements have been prepared by incorporating Zn, Mg, Si or Sr ions into β -TCP, α -TCP or nano-crystalline HA (Klammert *et al.* 2010, Pina *et al.* 2010, Lilley *et al.* 2005).

Hydrophilic polymers as xanthan gum, hydroxypropyl methylcellulose, polyacrylic acid and silica gel, or hyaluronic acid, albumin and fibrinogen are used as additives to improve injectability and cohesion of the cements (Flautre *et al.* 2003, Alkhraisat *et al.* 2009, Chauhan *et al.* 2006). On the other hand, collagen or other hydrophobic polymers such as poly(lactic acid-co-glycolic acid) are used to improve the mechanical properties of the cements (Tamimi *et al.* 2012).

Despite the huge number of articles, there are no systematic studies on the influence of the type of acid on the brushite cements formation and properties. The aim of this research was to study the formation of brushite cements starting from amorphous calcium phosphate double doped with Mg^{2+} and Zn^{2+} ions ((Mg, Zn)-ACP), and biocompatible carboxylic acids. For this purpose, citric, tartaric and lactic acids in different concentrations, various solid to liquid ratios and in presence of some additives were used. Phase composition, forming characteristics, morphology and *in vitro* behavior of the cements obtained were characterized by application of XRD, SEM and cell tests.

2. EXPERIMENTS

2.1. Cements preparation

Initial substances

Solid phase – Amorphous calcium phosphate, double doped with Mg^{2+} and Zn^{2+} ((Mg, Zn)-ACP). Laboratory-prepared by our method published earlier (Sezanova *et al.* 2016).

Liquid phase – Citric acid (CA) - 5% and 18% aqueous solutions; tartaric acid (TA) - 18% aqueous solution; and lactic acid (LA) - 18% aqueous solution.

Modifiers – Sodium alginate, 2% in respect of the solid phase; glycerin, 50% in respect of the liquid phase.

Technical preparation – The cement samples were prepared by mixing the solid and liquid phases and homogenizing for 6 - 15 min. The plastic mass formed was molded in rubber-molds with a diameter of 10 mm and a height of 5 mm, and then dried in air for 24 h for the XRD and SEM studies. A series of cement samples was prepared by variation of the solid to liquid ratio from 1:1 to 1:3.5 g/ml.

2.2. Cements characterization

Forming characteristics – the initial and final setting times of the cement samples prepared in the rubber-molds were determined by the Vicat needle method (Standard test method 1993).

X-ray diffraction analysis – The phase composition of the cements was determined on a D 500 (Germany) apparatus for XRD analysis, applying $CuK\alpha$ radiation and equipped with a secondary beam monochromator, within the 2θ range of $10-60^\circ$, a step of $0.02^\circ 2\theta$ and counting time of 30 s/step.

SEM images – The dried cylindrical cement molds were cut perpendicularly to the height of the cylinder and the surface of the rings was sputter-coated with gold. Their morphology and microstructure were observed using the scanning electron microscope JEOL JSM-5510 equipment.

2.3. In vitro testing

Test in SBF - The cement molds were kept up to 3 days in a fixed volume of conventional simulated body fluid (SBF, Kokubo (1990)) under static conditions. The composition of SBF was as follows: 142.0 mmol/l Na^+ , 5.0 mmol/l K^+ , 1.5 mmol/l Mg^{2+} , 2.5 mmol/l Ca^{2+} , 147.8 mmol/l Cl^- , 0.5

mmol/l SO_4^{2-} , 4.2 mmol/l HCO_3^- , 1.0 mmol/l HPO_4^{2-} , pH = 7.2. The pH of SBFc was periodically measured.

Cell tests - Only the cements prepared from (Mg, Zn)-ACP and tartaric acid were subjected to these tests. Three types of cells were used: (i) BALB/c 3T3 mouse fibroblasts; (ii) primary cultures from bone marrow cells; (iii) cultures (18-22 passages) from mouse bone explants.

The cells were grown in a D – MEM medium supplemented with 5-10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37 °C in a humidified CO_2 incubator (Antisel, Thermo Scientific, HEPA Class 100). For routine passages adherent cells were detached using a mixture of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA).

Integrated approach, including indirect and direct assessment of the impact of the material on cell adhesion, proliferation and survival was applied. For the indirect assessment, the cell culture was incubated for 3 or 6 days in the presence of the dry cements to obtain a modified culture medium. The viability of cells in this medium was determined by the MTT test after 72 h of cultivation. Direct assessment included determination of viability of murine bone marrow cells cultured in the presence of dry cements for 17 days. The number of viable cells was determined by the trypan blue dye exclusion method. Cell viability in direct and indirect assessment was expressed as a percentage of viable cells as compared to the control (cells cultured in non-modified medium where cell viability is 100%).

All experiments with laboratory animals, included in the present study, were performed in accordance with the Veterinary Medical Office in Bulgaria which follows the European Committee Standards concerning the care and use of laboratory animals (Registrations 25/26.01.2011 by the Regional Veterinary Medical Office, Sofia, and 11130127 by the National Veterinary Medical Office).

3. RESULTS AND DISCUSSION

3.1. Preparation and physico-chemical characterization of cements

The solid precursor used in this study was prepared by the method of biomimetic precipitation that allows obtaining of predetermined ion modified precipitates. In our case XRD amorphous calcium phosphate double doped with Mg^{2+} and Zn^{2+} ions, was prepared by continuous amorphous precipitation in a simulated body fluid (SBF) modified with these ions, at pH 8 (Sezanova *et al.* 2016). Then calcination of the precipitate at 400°C was performed. The obtained (Mg, Zn)-ACP solid precursor was characterized with molar ratios $(\text{Ca}^{2+} + \text{Mg}^{2+} + \text{Zn}^{2+})/\text{P} = 1.62$, $\text{Mg}^{2+}/(\text{Ca}^{2+} + \text{Mg}^{2+} + \text{Zn}^{2+}) = 0.09$ and $\text{Zn}^{2+}/(\text{Ca}^{2+} + \text{Mg}^{2+} + \text{Zn}^{2+}) = 0.03$ and a specific surface area of 26 m²/g.

Three hydroxy acids, namely citric acid (CA), tartaric acid (TA) and lactic acid (LA), were selected as biocompatible acids to form the liquid phase for cement preparation. In our previous work (Sezanova *et al.* 2014) we have shown that working with very concentrated acid solutions (more than 20%) is inappropriate, as it leads to acidification of the surrounding environment during *in vitro* experiments due to the release of unreacted acid. Thus we have limited the acid concentration up to 18%. We used a more dilute solution (5%) in the case of citric acid only. The experiments with all acids were performed by variation of the solid to liquid ratio from 1:1 to 1:3.5. The amount of liquid phase affects the rheology of the initially formed plastic mass, the possibility of complete wetting of the powder particles and the time needed for the phase transformation to occur.

The forming characteristics of the three series of obtained cements are presented in Table 1. The results illustrate the influence of the examined three acids on the setting times. The amount of liquid phase needed for manipulation of the cements depends on the acid type and strength. In the case of equal concentrations and solid to liquid ratios we have observed differences in both initial and final setting times. For each one of the three series, the shortest times refer to the cements prepared with 18% acid solutions and with the lowest amounts of liquid phase (molds 3CA, 1TA, 1LA). Both dilution and increasing in the amount of liquid acid phase increase the forming characteristics (time of manipulation, initial and final setting time) of the cements. This effect could be reduced by adding a

sorbing modifier (mold 6CA). Glycine modifier (mold 1TA) keeps the wet and thus decreases the solid to liquid ratio. Increased acid concentration shortens the initial and final setting times (molds 1CA and 5CD; molds 2CA and 7CA).

As longer the setting time is as the phase conversion is complete. Thus we have established full phase transformation for the molds 7CA, 3TA and 2LC (Fig. 1) with the longest setting times.

Table 1. Forming characteristics of brushite cements based on (Mg, Zn)-ACP

Mold	Liquid phase	Solid : Liquid	Additives in:		Mixing time, min	Initial setting time, min	Final setting time, min
			Solid phase	Liquid phase			
1CA	5% citric acid	1:2.1			10	15	60
2CA	5% citric acid	1:2.85			15	30	60
3CA	18% citric acid	1:1			6	8	12
4CA	18% citric acid	1:1.4			10	10	14
5CA	18% citric acid	1:2.1			15	21	48
6CA	18% citric acid	1:2.1	Sodium alginate		12	12	16
7CA	18% citric acid	1:2.85			15	22	50
1TA	18% tartaric acid	1:1		Glyce ryn	5	7	10
2TA	18% tartaric acid	1:2.1			10	20	27
3TA	18% tartaric acid	1:2.85			15	36	60
4TA	18% tartaric acid	1:2.85	Sodium alginate		9	9	12
1LA	18% lactic acid	1:2.85			3	3	5
2LA	18% lactic acid	1:3.5			15	30	60

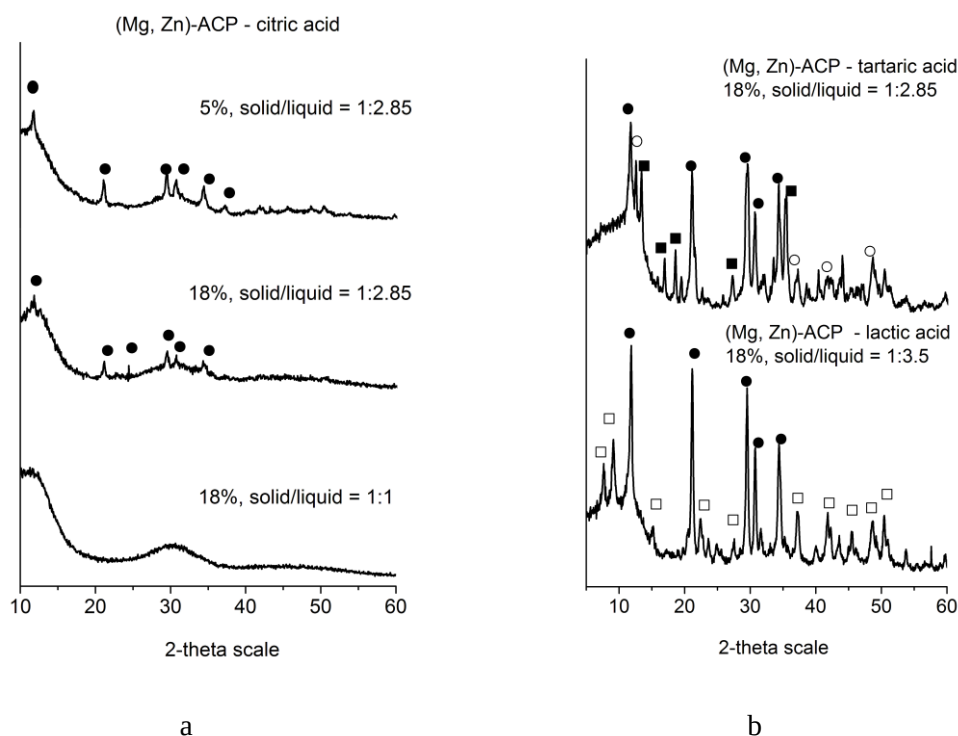


Fig. 1. X-ray powder patterns of cements prepared from (Mg, Zn)-ACP and carboxylic acids.
(a) Molds 3CA, 7CA and 2CA; (b) Molds 2LA and 3TA.

Marks: (●) – brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, DCPD); (■) – calcium tartrate tetrahydrate ($\text{CaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$);
(○) – calcium tartrate ($\text{CaC}_4\text{H}_4\text{O}_6$); (□) – calcium lactate ($\text{Ca}(\text{C}_3\text{H}_5\text{O}_3)_2$)

In the case of mold 3CA (18 % citric acid and 1:1 solid to liquid ratio) displaying the shortest setting times, there is no phase transformation and only the starting amorphous calcium phosphate were detected (Fig. 1a). For all three series of cements we have found that the increase in the solid to liquid ratio up to 1:2.85 leads to the appearance of brushite phase (molds 2CA, 7CA, Fig. 1a and molds 3TA, 2LA, Fig. 1b). In the X-ray patterns of cements with tartaric acid (mold 3TA, Fig. 1b) and lactic acid (mold 2LA, Fig. 1b), in addition to peaks of brushite, peaks belonging to low-soluble anhydrous calcium tartrate and calcium tartrate tetrahydrate, and to high-soluble calcium lactate are detected (Fig. 21b). The setting reaction of brushite cements (Dorozhkin 2011) consists of several stages: (i) dissolution of the solid precursor; (ii) formation of a super-saturated suspension; (iii) nucleation; and (iv) crystal growth. In all our experiments the dissolution/crystallization processes occurred in an acid medium. The initial (Mg, Zn)-ACP transformed partially or fully into less soluble salts, which are stable under these conditions. Möschner *et al.* (2009) reported that citric acid can be used to retard the hydration of Portland cement by slowing down the dissolution of the clinker grains. A similar effect was also observed in our experiments, that indicated incomplete conversion of the amorphous calcium phosphate in the presence of even 18% citric acid at a solid to liquid ratio of 1: 2.85.

SEM images of cement molds prepared from (Mg, Zn)-ACP and 18% solution of citric acid (CA), tartaric acid (TA) or lactic acid (LA) are presented in Fig. 2. For the molds in Fig. 2a (mold 7CA) and Fig. 2b (mold 3TA) the solid to liquid ratio was 1:2.85, in Fig. 2c (mold 2LA) it was 1:3.5 and in Fig. 2d (mold 1TA) it was 1:1. SEM images in Figs. 2a, 2b and 2c show typical brushite plate-like agglomerates of crystallites with different size. The results are in agreement with the results of the XRD analysis where partial transformation of ACP to brushite phase was registered in the case of the mold 7CA, and a complete transformation to brushite and respective salts in the case of the molds 3TA and 2LA (Fig. 1). Only the morphology of the 1TA mold (Fig. 2d) was significantly different. Probably, the smaller liquid phase allows only partial phase transformation of the ACP grains keeping their spherical habitus.

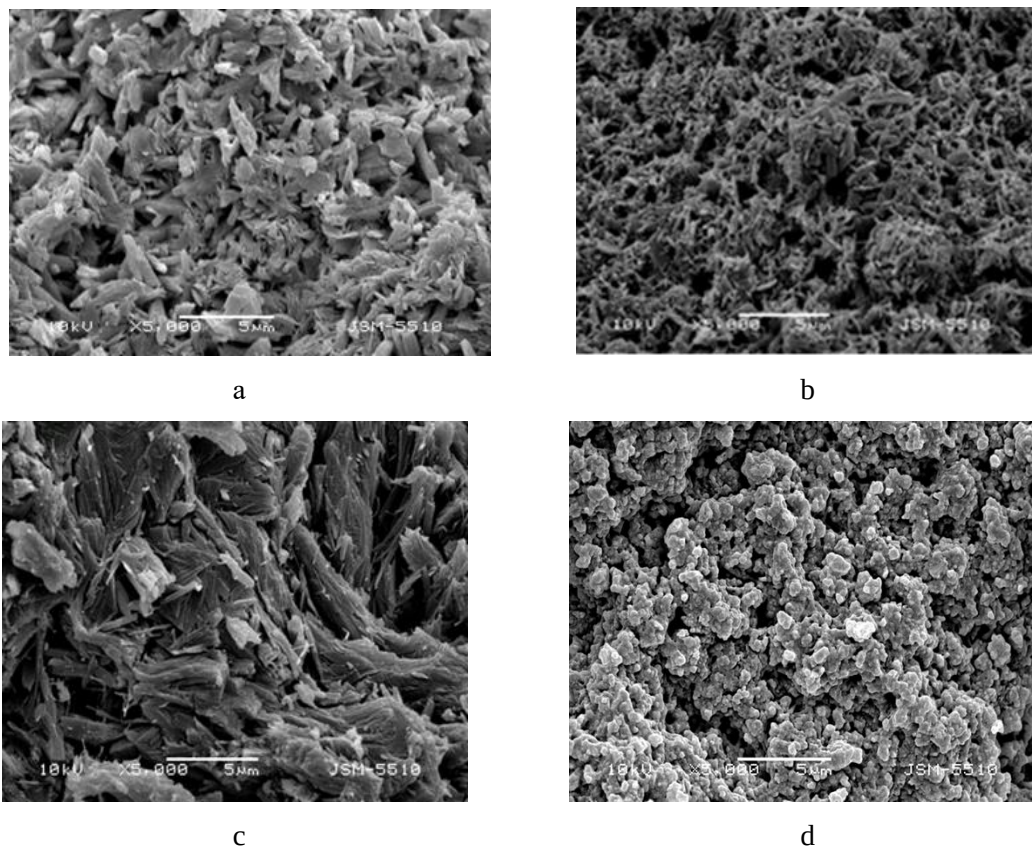


Fig. 2. SEM images of cement molds (Table 1): (a) 7CA (ACP+brushite (Fig. 1a)); (b) 3TA (brushite+calcium tartarate (Fig. 1b)); (c) 2LA (brushite+calcium lactate (Fig. 1b)) and (d) 1TA (no XRD data). All cement molds were prepared from (Mg, Zn)-ACP and 18% acid solution; for (a), (b) and (c) molds the amount of acid was dominated in comparison with the solid phase (Table 1).

3.2. *In vitro* testing

3.2.1. *Cements behavior in SBFc*

Electrolyte solutions with various compositions, referred to as simulated body fluids (SBFs), which claim to mimic the acellular human body plasma, have been proposed and used for testing the materials under biomimetic conditions.

We studied the behavior of cements based on (Mg, Zn)-ACP and 18% acids at different ratios in a conventional simulated body fluid, SBFc. Quick decrease (for 30 min) of the solution pH below the physiological one, followed by a slow decrease was registered for the citric acid molds 4CA and 5CA (Fig.3a) and for the mold 7CA (Fig.3b). This is probably due to the excess of citric acid in these cements where only partial phase transformation to brushite was observed (Fig.1a). In the case of citric acid cements with solid to liquid ratio of 1:1 where no phase transformation was detected (Fig.1a), the solution pH remained constant (pH 7.1). In contrary, the cement with 5% citric acid but solid to liquid ratio of 1:2.85 (mold 2CA) showed a small increase in pH (Fig.3a).

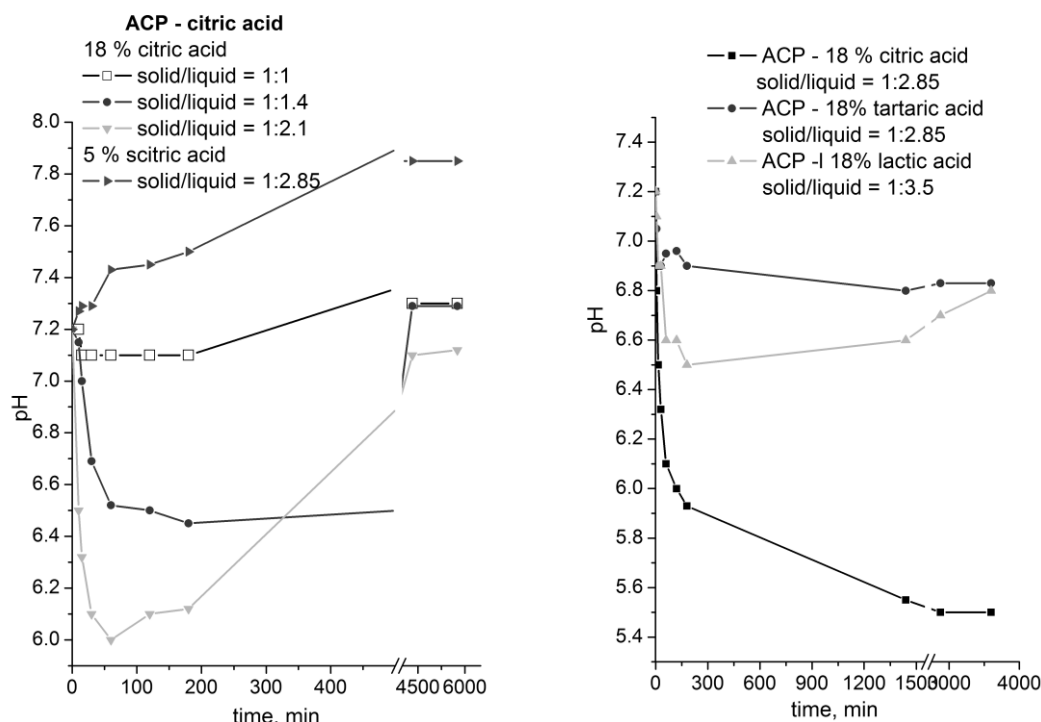


Fig. 3. Variation of the pH vs time dependence in the tests of cement molds behavior in SBFc: (a) molds 2CA, 3CA, 4CA, and 5CA (Table 1); and (b) molds 7CA, 3TA and 2LA (Table 1).

Cements prepared with 18% lactic acid and solid to liquid ratio of 1:3.5 (mold 2LA) also lowered the pH of SBF (Fig. 3b) but the effect was significantly weaker in comparison with citric acid. In this case the complete transformation of ACP to a mixture of brushite phase and high-soluble calcium lactate (Fig.1b) was the reason for the breakdown of the molds.

The best cement behavior in SBFc was displayed by the mold 3TA prepared with 18% tartaric acid (solid to liquid ratio 1:2.85) which kept the pH near to the physiological value. The reason is probably the complete transformation of ACP to a mixture of brushite phase and low-soluble calcium tartrate.

3.2.2. Cell tests

Only the cements prepared from (Mg, Zn)-ACP and 18 % tartaric acid were subjected to these tests. Three types of cells were used: (i) BALB/c 3T3 mouse fibroblasts; (ii) primary cultures from bone marrow cells; (iii) cultures (18-22 passages) from mouse bone explants.

BALB/3T3 is a non-tumorigenic cell line developed from disaggregated 14- to 17-day-old BALB/c mouse embryos. The cell cultures from mouse bone marrow and bone explants obtained as earlier described (Andonova-Lilova *et al.* 2012) from ICR mice (2-3 months old) of both sexes, were purchased from the Laboratory Animal Center (Slivnitsa, Bulgaria). Animals were given standard pellet diet and tap water *ad libitum*.

The relative cell viability, expressed as a percentage of the untreated control (100% viability), is presented in Fig. 4. By indirect assessment, the results showed a similar behavior of the three examined cell lines. Cell viability decreased after a 3-day incubation period, reaching its minimum of 45 % for cultures from mouse bone explants and primary cultures from bone marrow cells (BMC) (Fig. 4a). After a 6-day incubation period, cell viability was higher, reaching its maximum of 114 % for cultures from mouse bone explants.

Experiments were performed by the MTT test in the case of BALB/c 3T3 cells and cell cultures from mouse bone explants (Bone explants – Fig. 4a) and by the trypan blue dye exclusion technique in the

case of mouse bone marrow cells (BMC – Figs. 4a and 4b). The results obtained by the direct approach, when the cells were cultured directly on the material, revealed that the first 2-3 days of cultivation were accompanied by the experience of stress (the number of living cells was less than 50% as compared to the control) (Fig 4b). The number of living cells, however, gradually increased, and this tendency was enhanced after three days. The number of cells cultured in the presence of the material was maximal at the 4th day (~ 80% compared to control), then slowly decreased, but even on the 17th day, the number of living cells was still over 50% compared to control.

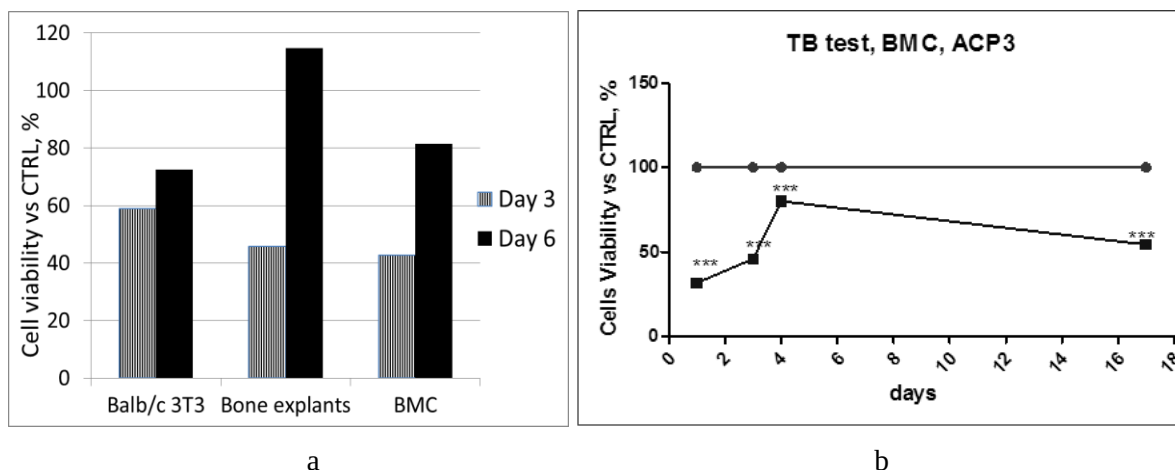


Fig. 4. Cell viability as a percentage of the untreated control by indirect (a) and direct (b) assessment.

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