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Influences of combined supplementation of calcium citrate and calcium carbonate on injectable and anti-washout hydroxyapatite/collagen bone paste utilizing sodium alginate

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Influences of combined supplementation of calcium citrate (Ca–Cit) and calcium carbonate ($CaCO_3$) on the hydroxyapatite/collagen (HAp/Col)–sodium alginate (Na–Alg) paste (HAp/Col paste) were investigated. Supplementation amounts of Ca–Cit and $CaCO_3$ were denoted as *Nx*, where *N* is a multiplication number to Ca^{2+} ion amounts for the equivalent reaction with Na–Alg obtained previously. Combined supplementation of 10*x* Ca–Cit and 2*x* $CaCO_3$ improved the anti-washout property at a washout ratio of 2.42 ± 0.72% and pH maintenance at 7.34 ± 0.08, these values were better than the best HAp/Col paste using Ca–Cit (5.91 ± 2.73% washout ratio and 6.72 ± 0.06 of pH). This is due to coordinate effects of initial washout inhibition by weak but rapid formation of long-range network by citric acid followed by long term anti-washout inhibition by strong but slow network formation by Ca^{2+} ions. The paste also showed good cytocompatibilty that MG63 cells proliferated with the culture time without any significant difference with the HAp/Col dense bodies. The HAp/Col paste is expected to be utilized in minimally invasive surgery of bone defect.

Key-words : Composites, Biomedical applications, Hydroxyapatite/collagen composite, Injectable bone paste, Calcium citrate, Sodium alginate

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1. Introduction

Autologous bone grafts are considered as the best by surgeons, even though they still face problems, e.g., donor site morbidity, deformity, scarring and chronic pain.[1)] Artificial bone void fillers based on bioactive ceramics are widely used and developed to replace or reduce the usage of autologous bones. Recently, clinical use of injectable bone pastes is increasing rapidly due to their advantages, ease to fit irregular-shaped defects without unexpected gaps between host and fillers,[2),3)] injectability to suit for minimally invasive surgery, which reduce patient discomfort and complications along with a decrease in health care costs. The most clinically successful pastes is so-called "apatite cement" that utilize the hydration hardening reaction by conversion of comparatively unstable calcium phosphates to hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, Hap] crystals.[4)] However, HAp remains in the patients' body due to its very low biodegradability, and its brittleness possibly lead a secondary bone fracture. In the sintered HAp, high porosity can solve the problems in part; however, it is difficult to apply to apatite cements, because addition of sufficient porogen decreases the operability and cause low fracture strength.

Application of biodegradable materials is the other solution for bone paste. Biodegradable bone void fillers clinically used are, e.g., porous β-tricalcium phosphate [β-$Ca_3(PO_4)_2$, β-TCP] and hydroxyapatite/collagen bone-like nanocomposite (HAp/Col). Sintered β-TCP is clinically used since 1970's due to it's potential of dissolution by body fluid and resorption by osteoclasts, which are the main reactions necessary to substitute with new bone. Thus, fabrication of chelate setting α- and β-TCP pastes using inositol-6-phosphate as a chelating agent and their potential in substitution with new bone have been reported.[5),6)] On the other hand, the HAp/Col was prepared by the simultaneous titration method and has similar bone like nanostructure, where *c*-axes of hydroxyapatite (HAp) nanocrystals were aligned along collagen fibrils. It is incorporated into bone remodeling process to substitute with newly formed bone when implanted into bone defect.[7),8)] Clinical trials on the porous HAp/Col proved to be a better material than porous β-TCP.[9)] An injectable HAp/Col paste had been developed using sodium alginate,[10)] which is already recognized as a biocompatible material with gelation ability by multivalent cations and a good lubricant;[11)] however,

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its anti-washout property was not sufficient for clinical use. To improve the anti-washout property, supplementation of various calcium compounds or organic acids were investigated;[12] however, only slight improvement was achieved by using low soluble calcium compounds, calcium carbonate ($CaCO_3$) and calcium citrate (Ca–Cit).

Recently Sato et al.[13] reported influences of excess supplementation of low soluble $CaCO_3$ or Ca–Cit for the improvement of anti-washout property. The HAp/Col pastes showed a washout ratio at less than 10% in mass by addition of Ca–Cit at 8 times or greater Ca^{2+} ion amounts than that for the equivalent reaction to Na–Alg with an observation of a slight decrease in the pH of the PBS after the anti-washout test. Further, a degradation of the paste prepared with supplementation of Ca–Cit at 20 times greater Ca^{2+} ion amounts than that for the equivalent reaction to Na–Alg was observed in Dulbecco's modified essential medium in 5 days. This result suggests that the formation of acid-induced alginate gel with high H^+ concentration inhibited eggbox structure formation. This phenomenon could be palliated by combined supplementation of acidic Ca–Cit and basic $CaCO_3$. In the present paper, influences of combined supplementation of Ca–Cit and $CaCO_3$ on injectable HAp/Col paste were investigated.

2. Materials and methods

2.1 Preparation of HAp/Col powder

The HAp/Col at a HAp and collagen mass ratio of 4:1 was prepared by a simultaneous titration of $Ca(OH)_2$ (prepared from the alkaline analysis grade $CaCO_3$, Wako pure chemicals Inc.) suspension and mixed solution of orthophosphoric acid (Reagent grade, Wako chemicals Inc.) and type-1 porcine dermal collagen (Biomaterial grade, Nitta Gelatin Inc.) with maintaining the water bath temperature at 40°C and pH of the reaction solution at 9.[8] The HAp/Col obtained was compacted into disks by squeezing water using a uniaxial press at 20 MPa, freeze dried and crushed into 100–212 μm in size with a ball mill (FRITSCH, Pulverisette, Germany) in vacuo using zirconia ball of 15 mmΦ. The powder obtained was then dehydrothermally crossed linked at 140°C for 12 h under a vacuum. In order to inhibit Ca^{2+} adsorption of the HAp/Col,[14] the powder was treated with 20 mM $CaCl_2$ solution for 3 days, filtered, freeze dried and stored at 4°C.

2.2 Characterization of HAp/Col powder

Inorganic phase of the HAp/Col powder was identified by the powder X-ray diffractometry (XRD, Rigaku, RINT-Ultima III) using Cu $K\alpha$ radiation from 2 to 60° of diffraction angle, 2θ, at a scanning rate of 2°/min. Inorganic and organic ratio of the HAp/Col powder was determined by the thermogravimetry-differential thermal analysis (TG–DTA, Rigaku, ThermoPlus, Japan) from room temperature to 1200°C at a heating rate of 5 °C/min. After the TG–DTA analysis, powder was identified by powder XRD and estimated Ca/P atomic ratio by calculation using relative intensity of 202 diffraction line of HAp (34.06°) and 220 diffraction line of TCP (34.34°).

2.3 Preparation of HAp/Col paste

The HAp/Col paste was prepared using the optimal conditions in Ref. 12, where the powder to liquid (P/L) ratio is 0.60 and mass ratio of HAp/Col powder and sodium alginate [Na–Alg, low viscosity (80–120 cP)], Wako pure chemicals, Inc.) is 9:1. The calcium compound additives chosen were $CaCO_3$ and Ca–Cit (Wako pure chemicals, Inc.) The reaction equivalent amount of Ca^{2+} ion to Na–Alg used in the experiments was 1.67 mmol per 1 g of Na–Alg reported in the Ref. 12. According to this, amounts of supplement was denoted as Nx, where N was multiplication number for the equivalent reaction amount of Ca^{2+} ion to Na–Alg. The HAp/Col paste was prepared by a mixing of the HAp/Col and Na–Alg aqueous solution with a combined supplementation of Ca–Cit and $CaCO_3$ under the conditions shown in **Table 1**, in which their abbreviations are also noted.

2.4 Viscosity test

Viscosity of the paste was measured using the method reported by Ishikawa et al.,[15] where 0.1 cm^3 of the paste was mixed for 3 min and a 2-kg glass plate was placed on the paste for 10 min after start of mixing. The spread area at 10 min after placing the glass was measured using its digital photograph with the Image-J program (version 1.48, NIH, USA).

2.5 Washout property test

Washout property of the HAp/Col paste was measured according to the procedure in Japanese Industrial Standard "JIS T 0330-4 Bioceramics- Part 4". The paste was prepared by mixing the raw materials for 3 min and was packed into a syringe of 4.8 mm in inner diameter and 16.5 mm in height. Within 5 min after mixing the paste was then squeezed onto a wire net with wire diameter of 0.5 mm and aperture of 2.0 mm. The paste was then soaked in 50 ml of phosphate buffered saline (PBS) and kept at 37°C in an incubator up to 72 h. A washout rate was calculated as the ratio of the paste on the wire net before and after soaking. The final pH of the solution was also measured.

2.6 Cytocompatibilty test

All paste materials were sterilized using ethylene oxide gas (EOG). The paste raw materials were mixed, molded into the shape of a cylinder of 7 mm diameter and 5 mm in height and were aged for 24 h in an incubator at 95 ± 5% RH to harden. A blank and HAp/Col dense body were chosen as controls. HAp/Col dense bodies was prepared by compact dehydration of the HAp/Col as synthesized into 5 mm in height and punched out into 7 mm diameter. They were freeze dried, dehydrothermally crosslinked and sterilized using EOG respectively. Before cell culture experiment, the HAp/Col dense bodies were soaked in

Table 1. Preparation conditions of the HAp/col paste with additives

HAp/Col (mg)	Na–Alg (mg)	H_2O (μl)	Ca–Cit		$CaCO_3$		Abbreviation	Final P/L ratio
			Times equivalent	Weight (mg)	Times equivalent	Weight (mg)		
170	18.9	264.4	8x	47.9	2x	6.30	8x-2x	0.79
			9x	53.9	1x	3.20	9x-1x	0.80
			10x	59.9	1x	3.20	10x-1x	0.82
			10x	59.9	2x	6.30	10x-2x	0.83
			12x	71.9	2x	6.30	12x-2x	0.87

x = Reaction equivalent amount of Ca^{2+} to Na–Alg (1.67 ± 0.07 mmol per 1 g of Na–Alg).

Dulbecco's Modified Eagle Medium (D-MEM) to adsorb Ca^{2+} ion and Mg^{2+} ions. MG-63 cells, derived from human osteosarcoma was used for the experiment. After a subculture of MG63 cells, 2×10^4 cells were seeded onto each well in a 6 well tissue culture treated polystyrene plate (TCPS). One day after seeding, the paste and control samples were placed in each well and cultured for 7 days. Medium was changed every 2 days. After desired time interval, the cells were detached by trypsin/EDTA and the cell numbers were calculated using hemocytometer. The used media was collected during the medium change to measure the Ca^{2+} and PO_4^{3-} ion concentrations using an inductively coupled plasma-atomic emission spectrometer (SPS7800, SII NanoTechnology, Japan; ICP-AES).

2.7 Statistical analysis

The results are shown as the mean ± standard deviation. Statistical analysis was performed using one way analysis of variance with Turkey-HSD post hoc test. The statistical significance was set at $p < 0.05$.

3. Results and discussion

The mass ratio of inorganic phase to total mass of the dried HAp/Col calculated from TG–DTA measurement was 80.2%, which was similar to the ratio of starting materials as described in the previous report.[8] **Figure 1** shows the XRD patterns of as prepared HAp/Col and heated treated HAp/Col. Powder X-ray diffraction pattern of the as-prepared HAp/Col showed that the inorganic phase of the HAp/Col was low crystalline HAp. A part of the low crystalline HAp decomposed to β-tricalcium phosphate [β-$Ca_3(PO_4)_2$, TCP] by heating at 1200°C, i.e., the low crystalline HAp was calcium deficient HAp. Mass ratios of crystalline phases of heat treated HAp/Col quantified from the XRD peaks was 84.5% of HAp and 15.5% of TCP, and a Ca/P atomic ratio of the inorganic phase of HAp/Col was 1.63.

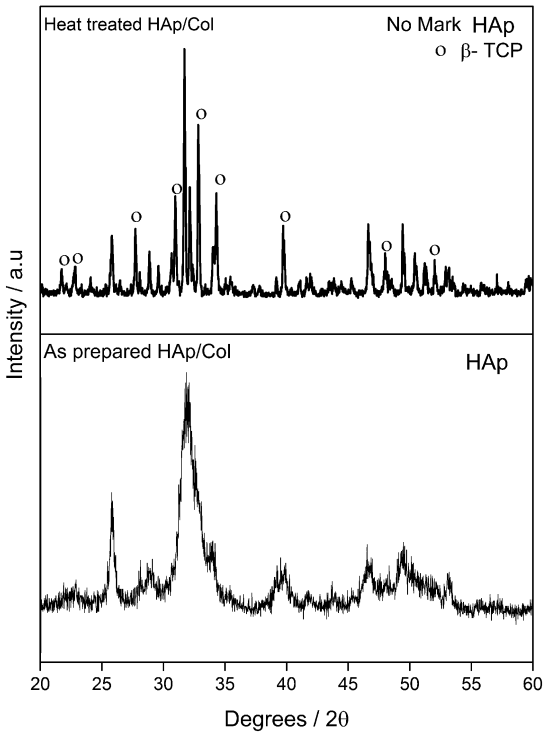

Fig. 1. XRD patterns of as prepared HAp/Col and the HAp/Col after heated at 1200°C.

Spread area of the paste combination supplemented with Ca–Cit and $CaCO_3$ increased with Ca^{2+} amounts as shown in **Fig. 2**. Further, spread areas of the pastes supplemented with totally 10*x* Ca^{2+} ion, approximately 120–130 mm^2, was very similar to that of the paste solely supplemented with 10*x* Ca–Cit as reported by Sato et al.[13] These results suggested pastes supplemented with calcium compounds had higher influences on viscosity. Effects on initial viscosity with acid compounds as supplements were low in comparison to those of Ca^{2+} amounts. This could be caused by difference between strong eggbox gel and weak acid-induced gel. Anti-washout property became better with increasing in total Ca^{2+} amounts up to 10*x*-2*x* and again worse at 12*x*-2*x* as shown in **Fig. 3**. The anti-washout ratios for the paste with

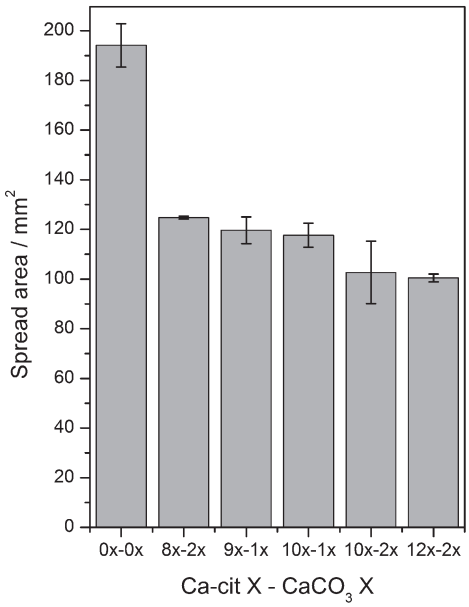

Fig. 2. Spread area of the HAp/Col paste combination supplemented with Ca–Cit and $CaCO_3$. Data represent mean ± standard deviation (SD) for $n = 3$.

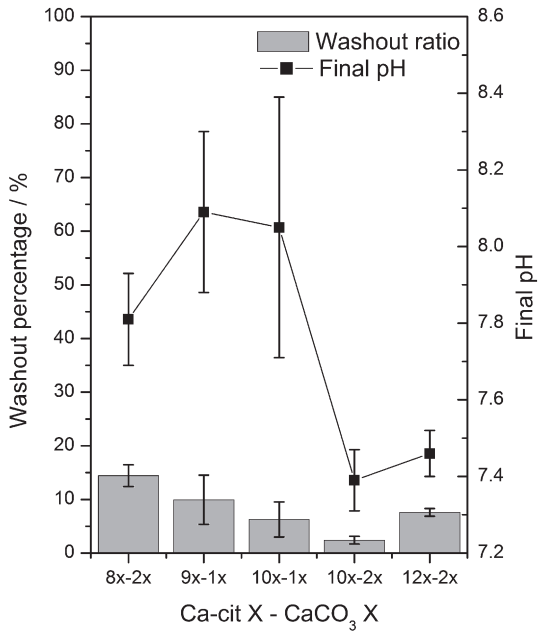

Fig. 3. Washout behavior and final pH of the medium after 72 h. Data represent mean ±SD for $n = 3$.

combined supplementation demonstrated a smaller value at an amount of 10*x*-2*x* (2.42 ± 0.72%) in comparison to the 10*x* of Ca–Cit solely supplemented pastes (5.91 ± 2.73%).[12] Further, the final pH of the solution was maintained as the original pH for the 10*x*-2*x* (7.34 ± 0.08) compared with the 10*x* of Ca–Cit supplemented paste (6.72 ± 0.06);[12] though final pH of 10*x*-1*x* and 9*x*-1*x* increased.

In our previous reports,[12),13)] to improve the anti-washout property of HAp/Col paste, the paste was supplemented with organic acid or calcium compounds, resulting in increase of viscosity and slight extension of washout time. The mechanisms for the increase in viscosity were different in each supplement; i.e., acids increased viscosity of alginate by acidic environment and calcium compounds by crosslinkage of alginate via Ca^{2+} ions. The pastes prepared with high soluble calcium compound showed no improvements in anti-washout property, because high soluble calcium compounds discharge Ca^{2+} ions quickly to form short range gel near the calcium compounds, which increased in paste viscosity and inhibits the diffusion of Ca^{2+} ions to form long-range network to prevent the washout. Contrarily, the pastes prepared with low soluble calcium compound, $CaCO_3$ and Ca–Cit improved the anti-washout property. However, paste supplemented with 20*x* $CaCO_3$ decayed completely within 72 h and did not acquire sufficient wash out property. Large amounts of $CaCO_3$ allowed formation of eggbox gel to increase viscosity; however, the gel formation by low Ca^{2+} concentration due to low solubility of $CaCO_3$, might be insufficient for the anti-washout in PBS by substitution of Ca^{2+} to Na^+, which leads to the decomposition of alginate gel network. Contrarily, Ca–Cit supplementation showed anti-washout property because of alginate gelation by acidic pH. Acidic alginate gel might be stronger than the gel formed by small amounts of Ca^{2+} under PBS condition. In addition, Ca–Cit in the paste also formed the egg-box structure gradually by large amount of Ca^{2+} to reinforce the paste. However, the pH of the PBS after the anti-washout test became acidic, which can compromise the biocompatibility of the paste.

Similar reactions occurred in the paste with combined supplementation of Ca–Cit and $CaCO_3$. First, Ca–Cit, comparatively higher solubility than $CaCO_3$, dissolved in the paste and allowed to form weak acid-induced gel. Dissolution of $CaCO_3$ was followed by acid environment and increased the pH, thus weakening the acid-induced gel. In the meantime, Ca^{2+} ions started to interact with gelation sites of alginate due to increase of free Ca^{2+} ions from chelation with citrates; this meant that the reason of small improvement in the wash out property by the solely supplementation of Ca–Cit[12] could not only be by inhibition of the eggbox formation by acid-induced gel but also by inhibition of interaction between Ca^{2+} ions and the eggbox sites. This is due to competitive reaction between the chelation by citrate and the eggbox formation.

Supply of Ca^{2+} ions from $CaCO_3$ less than or equal to 10%, the amount of Ca^{2+} ions might not be sufficient for moving reaction equivalent to the eggbox structure formation; thus, the free Ca^{2+} ions leached from the paste and increased the pH. Contrarily, Ca^{2+} ions supplied from $CaCO_3$ more than 10%, were used for the formation of eggbox structure as well as chelating with citrates; therefore, pHs were comparatively neutral than small Ca^{2+} ions supplied from $CaCO_3$. Further, initial acid-induced gel formation was also important for anti-washout property; thus, the 10*x*-2*x* paste showed higher washout percentage than others. Increasing in the washout percentage for the 12*x*-2*x* paste could be due to the slight low $CaCO_3$ ratio than the 10*x*-2*x* paste. Summarizing the washout test, supplementation of $CaCO_3$ amount appropriately,

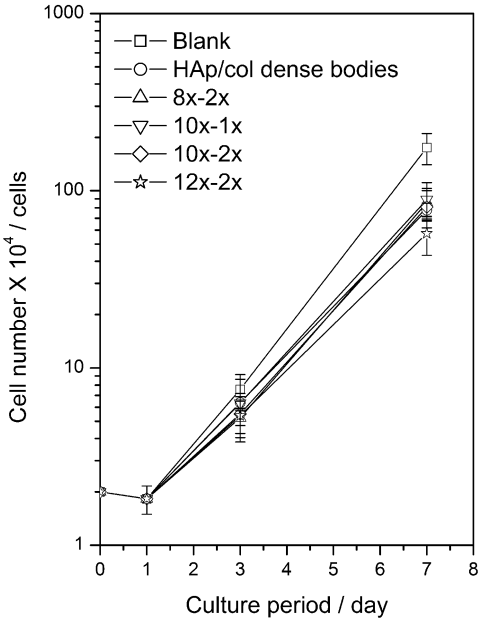

Fig. 4. In vitro analysis of the combination of additives on HAp/Col paste. Data represent mean ± SD for $n = 5$.

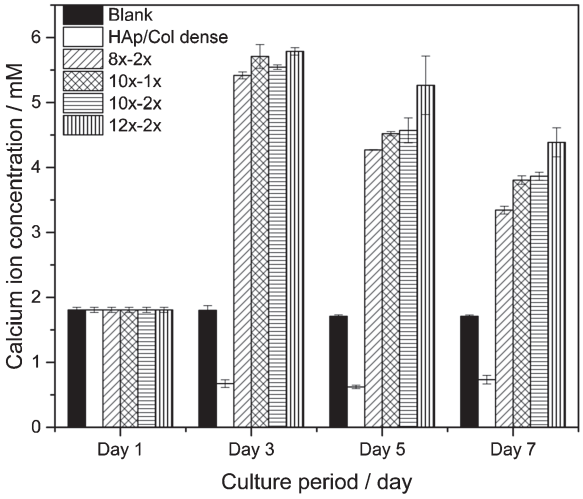

Fig. 5. Calcium ion concentration of the culture medium after the cell culture test. Data represent mean ± SD for $n = 3$.

i.e., respective Ca–Cit and $CaCO_3$ amount of 10*x* and 2*x*; and 12*x* and 2*x*, improved the anti-washout property without impairing the pH of the PBS.

Figure 4 shows the cell proliferation curve for the MG-63 cells cultured with the HAp/Col pastes. The 9*x*-1*x* paste was not used for the cell culture test because the pH of the medium turned basic after 72 h. Significant difference between the test groups and TCPS group were found for each measurement. All four combinations showed good proliferation activity without any significant difference compared to the HAp/Col dense bodies, which shows very good biocompatibility in vivo.[7]

Figure 5 and **6** show the changes in the Ca^{2+} and PO_4^{3-} ion concentrations in the culture medium. At day 3, the culture media of test groups contained 3 times higher Ca^{2+} ion concentration than control TCPS due to the gradual release of Ca^{2+} ions from $CaCO_3$ and Ca–Cit. But, the ratio compared to the control decreased as the culture period increased. In contrast, the Ca^{2+} ion

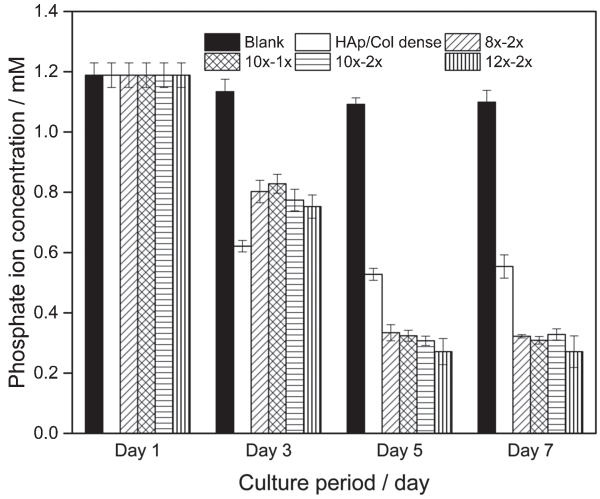

Fig. 6. Phosphate ion concentration of the culture medium after the cell culture test. Data represent mean ± SD for $n = 3$.

concentration decreased in HAp/Col dense body, due to the adsorption of Ca^{2+} ions on HAp/Col as reported by Sotome et al.[14] The concentration of PO_4^{3-} ions continuously decreased with the culture period. The PO_4^{3-} ion concentration in the culture media of test groups and HAp/Col dense body was low compared to control TCPS. Changes in Ca^{2+} and reduction in PO_4^{3-} ion concentration in the culture medium could affectthe proliferation and differentiation of almost all cells; however, no quite suppression in the cell proliferation observed in the test and HAp/Col dense body group, which demonstrated that these ion concentration changes had no critical effects on cell viability and proliferation. The cytocompatability of the pastes is as good as the HAp/Col, clinically used material in Japan.

These results suggest that the influence of the combined supplementation of Ca–Cit and $CaCO_3$ on the injectable HAp/Col paste improved the anti-washout property and pH control ability without critical influences on biocompatibility.

4. Conclusions

Combined supplementations of Ca–Cit and $CaCO_3$ improved the anti-washout property and pH controllability of the injectable HAp/Col paste. The improvements were caused by a competitive reaction occurred coordinately in the pastes. From in vitro cell culture studies, all combinations showed good cytocompatibility without any significant suppression of cell proliferations. Hence, the presently prepared HAp/Col pastes could be good candidates for injectable artificial bone, which has the potential for incorporation into bone remodeling process.

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