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Non-decay type fast-setting calcium phosphate cement using chitosan

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A non-decay type fast-setting calcium phosphate cement (nd-FSCPC) has been described, which did not decay but set within approximately 5–6 min even when the paste was immersed in serum immediately after mixing, and which forms hydroxyapatite as its end product. nd-FSCPC was produced by adding sodium alginate to the liquid phase of the base cement FSCPC. Sodium alginate forms a water-insoluble gel, and reduces the process of fluid penetration into the paste which is the cause of decay. The aim of this investigation was to confirm the mechanism of the non-decaying behaviour of nd-FSCPC proposed in a previous paper, using another chemical with properties similar to those of sodium alginate. Also, it was intended to further improve both the mechanical properties and tissue response of nd-FSCPC. Chitosan, which also forms a water-insoluble gel in the presence of calcium ions and has been reported to have pharmacologically beneficial effects on osteoconductivity, was added to the liquid phase of the base cement FSCPC. The cement thus prepared showed behaviour similar to that of nd-FSCPC using sodium alginate. The cement paste did not decay but set within approximately 5–6 min even when immersed in serum immediately after mixing. DTS value of the set mass was approximately 3–4 MPa, slightly lower than that of nd-FSCPC using sodium alginate, and no inhibitory effect was observed for the transformation of cement component to apatite within the range used in this investigation (up to 1.5%). Therefore, it was concluded that the mechanism of non-decaying behaviour was, at least in part, reduction of fluid penetration into the cement paste. nd-FSCPC using chitosan showed slightly poorer mechanical properties than that using sodium alginate. However, pharmacological effects such as osteoconductivity could be expected in nd-FSCPC using chitosan. Thus, this cement may be useful as a more sophisticated bioactive cement than nd-FSCPC using sodium alginate.

1. Introduction

Non-decay type fast-setting calcium phosphate cement (nd-FSCPC) is a promising bioactive cement which does not decay but sets within approximately 5–6 min even when the paste is immersed in serum immediately after mixing to form hydroxyapatite (HAP: $Ca_{10}(PO_4)_6(OH)_2$) only as a final product, and thus can be used in surgical procedures where exposure of cement to blood often occurs and complete hemostasis is sometimes very difficult [1, 2]. nd-FSCPC consists of powder and liquid phases the former of which is the same as conventional CPC (c-CPC) and FSCPC, an equimolar mixture of tetracalcium phosphate (TTCP:$Ca_4(PO_4)_2O$) and dicalcium phosphate anhydrous (DCPA:$CaHPO_4$) or dicalcium phosphate dihydrate (DCPD: $CaHPO_4{\cdot}2H_2O$) [3–8], while the latter is a neutral phosphate (0.2 mol/l) containing 0.5% sodium alginate. In contrast to nd-FSCPC, c-CPC and FSCPC decay completely when the paste is immersed in serum immediately after mixing, the decay occurring much faster in c-CPC than FSCPC. According to Miyamoto *et al.* [2], two independent processes occur when the cement paste is immersed in fluid as shown in Fig. 1. One is the formation of HAP which is the key step of the cement setting reaction (right-hand side) [7, 8], a process which begins when the cement is moistened. The other is the penetration of fluid into the cement paste, which induces decay. The behaviour of cement is determined as a net result of these processes. FSCPC shows accelerated HAP formation [7, 9, 10], and as a result, has a setting time of approximately 5 min in contrast to 30–60 min for c-CPC. Both cements, c-CPC and FSCPC, are stable, even in fluid, once set. However, FSCPC still decays when the paste is immersed into serum immediately after mixing; i.e. the setting reaction is slower than the decay process even in FSCPC. nd-FSCPC was developed to control the fluid penetration process (left-hand side) in addition to retaining the accelerated HAP formation (right-hand side). The fluid penetration process was reduced by sodium alginate, a compound which

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CPCs	Fluid penetration ← Decay	Cement paste	HAP formation → Setting	Properties: Setting time (min)	Properties: Behaviour
Conventional (c-CPC)	No response		No response	30–60	Decay
Fast-setting type (FSCPC)	No response		Acceleration (Neutral phosphate)	5	Decay
Non-decay type (nd-FSCPC) using sodium alginate	Reduction (Sodium alginate)		Acceleration (Neutral phosphate)	5–7	Set
Non-decay type (nd-FSCPC) using chitosan	Reduction (Chitosan)		Acceleration (Neutral phosphate)	5–7	Set

Figure 1 Illustration of the reaction of cement paste when immersed in fluid with some properties of cements. Two independent reactions proceed competitively. Right-hand side: setting reaction of cement which is closely related to cement conversion to hydroxyapatite. Left-hand side: decay reaction induced by the penetration of fluid into cement paste.

becomes a water-insoluble gel, calcium alginate, in the presence of calcium ions which may be supplied by the dissolution of TTCP and/or DCPA. As a result, nd-FSCPC did not decay but set within approximately 5–6 min even when the paste was immersed in fluid such as distilled water or serum immediately after mixing. Ishikawa *et al.* [1] chose sodium alginate to reduce the process of fluid penetration into the cement paste. They stated the requirements of the additives for a non-decay type cement as follows:

1. It should inhibit the decay of cement paste in liquid.
2. It should not inhibit the conversion of cement to HAP.
3. It should not decrease the handling properties of the cement paste.
4. It should not decrease the mechanical properties of the set cement.
5. It should show excellent biocompatibility, at least comparable to that of apatite.
6. It should be adsorbed within a relatively short time.

Sodium alginate is a good additive since it forms a water-insoluble calcium alginate gel in the presence of calcium ions and is known to be adsorbed well *in vivo* [11–16]. However, it is not the only chemical which fits the requirements outlined above. If the mechanism to obtain non-decay behaviour is correct, there should be other additives which show better mechanical properties or tissue response. Indeed, the DTS value of nd-FSCPC when the paste was immersed in serum immediately after mixing was half that when the paste was kept at 100% humidity [1, 2]. This indicates that reduction of fluid penetration process by sodium alginate is not perfect.

Chitosan is a candidate additive. Chitosan also forms a water-insoluble gel not only in the presence of calcium but also with several other cations. Chitosan may form a better water-insoluble gel and produce a greater reductive effect on the fluid penetration process compared with sodium alginate. An additional important factor is the pharmacological effect of chitosan. Certain modified chitosans possess properties that promote ordered regeneration of soft tissues and osteoconduction [17, 18]. It has been demonstrated *in vivo* that endochondral ossification and direct membranous osteoinduction are induced by chitosan [19–22]. In addition, due to its N-acetyl-glucosamine repeating units, chitosan has some similarity with glucosaminoglycans and binds to growth factors such as fibroblast growth factor (FGF) [23] normally present in the osseous trabecular tissue, and has also been shown to confer mitogenic activity on various types of mesenchymal cells including osteoblasts [24]. Chitosan activates macrophages and monocytes, thus inducing production of FGF and platelet-derived growth factor (PDGF) [17], favouring the histoarchitectural organization of the osseous tissue [25].

2. Materials and methods

2.1. Preparation of calcium phosphate cements (CPCs)

The original powder of the CPCs was prepared as described previously [1, 5–7, 10]. Briefly, an equimolar mixture of TTCP and DCPA was made with a micromill (SK-M2; Kyoritsu Riko, Tokyo, Japan) and stored in a vacuum desiccator at 60 °C. The liquid phase was made by mixing equal concentrations (0.2 mol/l) of disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) so that the pH of the solution became pH 7.4 at 37 °C. The resultant solution has a chemical formula of approximately $Na_{1.8}H_{1.2}PO_4$, and this formula will be used for simplicity in the following text. In the case of nd-FSCPC using chitosan, chitosan (PC-100, Ajinomoto, Tokyo, Japan) was further dissolved in 0.2 mol/l $Na_{1.8}H_{1.2}PO_4$. No adjustment was made for the concentration of neutral phosphate due to the addition of chitosan, since the volume of chitosan was negligible even at the maximum concentration used in the present investigation, i.e. 1.5 wt %.

2.2. Percentage of remaining cement

Percentage of remaining largest cement mass after 24 h immersion in calf serum (Gibco Brl, NY, USA) was measured for the quantitative evaluation of decay behaviour. This value is dependent on the shape and size of the cement paste along with immersion conditions. Therefore, we employed the same procedure as reported previously [1, 2]. Cement powder was mixed with liquid phase at a powder to liquid ratio of 3.5 (g/ml), and then packed in a cylindrical mould so that the paste samples were 4.7 mm in diameter and 8 mm in height. The mould was made by cutting off the front portion of a 1 cm^3 plastic syringe (Terumo, Tokyo, Japan). A force of approximately 3 kg per 4.7 mm diameter was applied for packing the cement into the moulds. After smoothing the surface of the cement on the open side, the piston of the syringe was pushed so that the paste was immersed in serum maintained at 37 °C. After 24 h immersion, the largest mass of cement was collected and washed with distilled water, followed by freeze-drying (FD-1, Tokyo Rikakiki Co., Tokyo, Japan). The percentages of remaining cement used were averages of at least five specimens. The bars in figures denote standard deviation.

2.3. Setting time of the cement

The setting time of the cement was measured in serum kept at 37 °C, basically according to the international standard ISO 1566 for dental zinc phosphate cements. In this method, the cement is considered set when a 400 g weight loaded onto a Vicat needle with a tip diameter of 1 mm fails to make a perceptible circular indentation on its surface. The standard requires that the cement be maintained at a temperature of 37 °C and relative humidity of at least 37%; in the present investigation, the conditions were 37 °C and cement paste was immersed in serum [1, 2]. Also, setting time was measured at 37 °C and relative humidity of 100%. Setting times used were the average values of at least five specimens.

2.4. Mechanical strength measurements

Mechanical strength of set CPCs was evaluated in terms of wet diametral tensile strength (DTS). Cement paste was packed in a manner similar to that for the measurement of percentage remaining cement. A plastic tube with an inner diameter of 6 mm and a plastic rod with a diameter of 6 mm were used to make paste samples 6 mm in diameter and 3 mm in height. Cement paste was then immersed in serum by pushing the piston. After immersion in the serum for 24 h, cement mass, if any remained, was taken out of the serum and used for wet DTS measurement. The diameter and length of each specimen were measured with a micrometer. The samples were crushed with a crosshead speed of 1.0 mm/min using a universal testing machine (AGS-500A, Shimadzu, Kyoto, Japan). The DTS values used were averages of at least ten specimens. The bars in figures denote standard deviation.

2.5. X-ray diffraction analysis

Compositions of CPCs before and after immersion in serum were identified by means of X-ray diffraction analysis (XRD). After the specimens were removed from the serum following immersion for 24 h, they were immediately quenched in liquid N_2, lyophilized and freeze-dried (Automatic Freeze-Dryer 10-010, Virtis Co., Gardiner, NY). The freeze-dried samples were ground into fine powders and characterized by XRD. The XRD patterns of the specimens were recorded with a vertically mounted diffractometer system (ADG-301, Toshiba, Tokyo, Japan) using Ni-filtered CuK_α radiation ($\lambda = 0.1540$ nm) generated at 30 kV and 10 mA. The samples were first scanned from 3 to 60 degrees in 2θ (where θ is the Bragg angle) to determine the reaction product in continuous mode (1.0° 2θ/min, time constant 2 s) on a strip-chart recorder.

3. Results

Fig. 2 shows the behaviour of CPCs when the pastes were immersed in serum at 37 °C immediately after mixing. c-CPC and FSCPC decayed almost completely, whereas both nd-FSCPCs which contain sodium alginate and chitosan did not decay but set in serum. Table I summarizes the setting times of nd-FSCPC with various concentrations of sodium alginate and chitosan. c-CPC and FSCPC decayed upon immersion into serum, and thus setting time could not be measured. The setting times of cements at 37 °C and 100% humidity are also listed for comparison. Fig. 3 summarizes the effects of chitosan and sodium alginate on the percentage remaining cement. Both compounds were added to the liquid phase of the base cement FSCPC. The concentrations of the chemicals shown are those in the liquid phase and the mixing ratio of powder to liquid was 3.5 (g/ml). With the addition of the sodium alginate or chitosan, percentage remaining cement increased to 100% at 0.4% chitosan and 0.5% sodium alginate, respectively. The DTS values of cements when the paste was immersed in serum immediately after mixing and kept for 24 h are shown in Fig. 4. Cement containing less than approximately 0.4% chitosan and less than 0.5% sodium

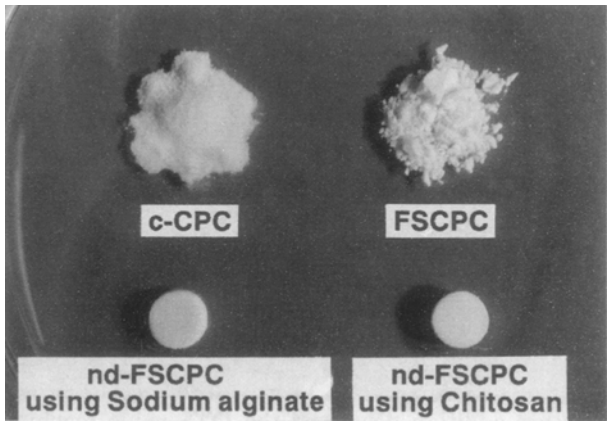

Figure 2 Behaviour of CPCs when pastes were immersed in serum at 37 °C immediately after mixing and kept there for 24 h.

TABLE I Setting time of non-decay type fast-setting calcium phosphate cements with various concentrations of sodium alginate or chitosan when fast-setting calcium phosphate cement (FSCPC) were used as base cements. The cement was mixed at a powder to liquid ratio of 3.5 and immersed in serum immediately after mixing. Setting time of FSCPC and c-CPC is shown for comparison.

CPCs	Chitosan (wt %)[a]	Sodium alginate (wt %) [a]	Setting time (min)	
			In serum	100% humidity[b]
c-CPC	–	–	Decay[c]	31.3 ± 1.9
FSCPC	–	–	Decay[c]	5.3 ± 0.3
nd-FSCPC	0.40	–	6.3 ± 0.3	5.3 ± 0.3
nd-FSCPC	0.50	–	6.2 ± 0.3	5.7 ± 0.4
nd-FSCPC	0.80	–	6.3 ± 0.4	6.0 ± 0.5
nd-FSCPC	1.00	–	5.7 ± 0.5	5.1 ± 0.2
nd-FSCPC	1.50	–	5.7 ± 0.5	5.4 ± 0.4
nd-FSCPC	–	0.25	6.2 ± 1.2[d]	5.2 ± 0.4[ef]
nd-FSCPC	–	0.50	7.0 ± 1.6[d]	5.5 ± 0.5[eg]
nd-FSCPC	–	1.00	6.3 ± 1.3[d]	5.3 ± 0.5[e]
nd-FSCPC	–	1.50	6.5 ± 1.5[d]	5.2 ± 0.4[e]
nd-FSCPC	–	2.00	6.2 ± 1.2[d]	5.5 ± 0.5[e]

[a] Concentration in the liquid phase.
[b] Setting time of the cement when the paste was free from serum but kept at 37 °C and 100% humidity.
[c] Paste decayed upon immersion in serum, thus setting time could not be measured.
[d] Cited from [2].
[e] Cited from [1].
[f] Concentration in [1] is 0.2%.
[g] Concentration in [1] is 0.6%

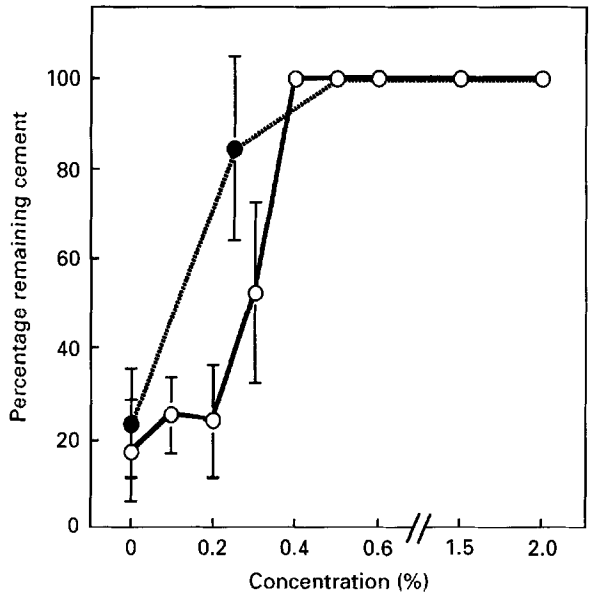

Figure 3 Effects of sodium alginate and chitosan on the percentage of remaining cement paste after 24 h. The cement paste was immersed in serum at 37 °C immediately after mixing (mixing ratio of 3.5, powder/liquid), and FSCPC was used as base cement; ○, nd-FSCPC using chitosan; ●, nd-FSCPC using sodium alginate. Some data taken from a previous paper [2].

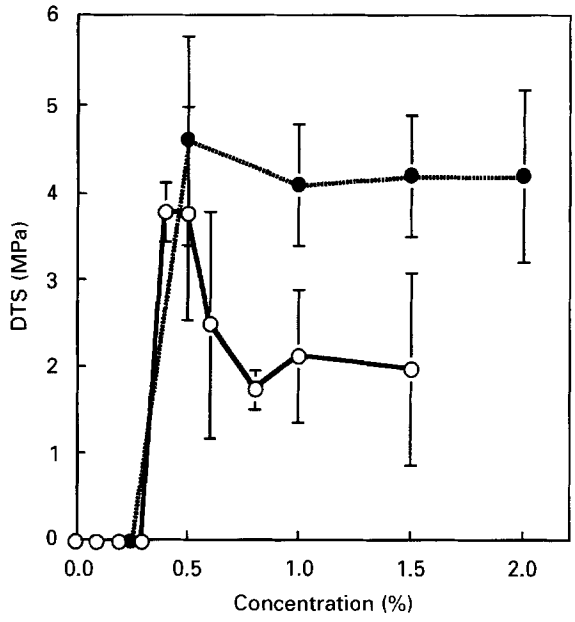

Figure 4 Effects of sodium alginate and chitosan on mechanical strength (wet diametral tensile strength (DTS value) of cement when the paste was immersed in serum (37 °C) immediately after mixing and kept in serum for 24 h: ○, nd-FSCPC using chitosan; ●, nd-FSCPC using sodium alginate. Some data taken from a previous paper [2].

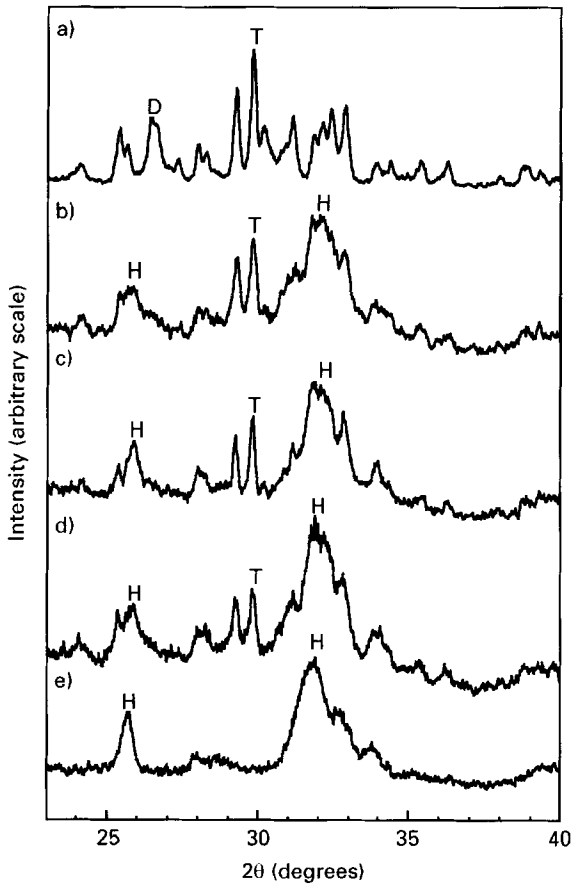

Figure 5 Powder X-ray diffraction patterns of FSCPC and nd-FSCPC using sodium alginate and chitosan, kept in serum at 37 °C for 24 h. The powder phases of calcium phosphate cement and poorly crystallized HAP are shown for comparison (TTCP = tetracalcium phosphate; DCPA = dicalcium phosphate anhydrous; T – TTCP; D – DCPA; H – HAP). (a) CPC powder; (b) FSCPC; (c) nd-FSCPC using sodium alginate; (d) nd-FSCPC using chitosan; (e) HAP.

alginate decayed, and thus DTS values were defined as zero. Maximum DTS value was observed when 0.4–0.5% chitosan was added to the liquid phase. Further addition decreased the DTS value reaching approximately 2 MPa when liquid phase of nd-FSCPC contained 1.5% chitosan. Fig. 5 shows the

XRD patterns of base cement, FSCPC and nd-FSCPC using sodium alginate and chitosan after being kept in serum for 24 h. FSCPC decayed completely, and the decayed powder was collected and used for XRD measurement. Also, the powder phase of nd-FSCPC, an equimolar mixture of TTCP and DCPA, is shown in Fig. 5 with poorly crystallized HAP for comparison.

4. Discussion

Fabrication of nd-FSCPC was possible using chitosan instead of sodium alginate with FSCPC as the base cement. Fig. 2 clearly shows the differences between nd-FSCPCs and other CPCs, c-CPC and FSCPC, when the pastes were immersed in serum immediately after mixing. c-CPC and FSCPC decayed whereas nd-FSCPC did not and set regardless of the addition of either sodium alginate or chitosan. The lack of a significant difference in the behaviour of nd-FSCPCs using sodium alginate or chitosan indicated that both compounds play the same roles during the setting reaction in serum. These results support the proposed mechanism of decay prevention; reduction of fluid penetration by formation of a water-insoluble gel [1, 2]. Sodium alginate forms calcium alginate and chitosan forms calcium–chitosan complex. The degree of reduction of fluid penetration, of course, is dependent on the kind of gel formed. One of the aims of this study was to prepare a superior nd-FSCPC. Unfortunately, nd-FSCPC using chitosan showed poorer mechanical properties than that using sodium alginate. For example, the percentage remaining cement was lower in the cement containing chitosan than in that containing sodium alginate at the same concentration (Fig. 3). Also, nd-FSCPC using chitosan showed lower mechanical strength as shown in Fig. 4. As stated above, setting reaction and fluid penetration proceed independently. Since setting times of the nd-FSCPC using sodium alginate and chitosan were the same, approximately 5–7 min, the inhibitory effects on HAP formation, i.e. setting reaction, by sodium alginate or chitosan seem to be minimum within the concentration range used in this study. Therefore, percentage remaining ratio would be determined by the degree of reduction in the fluid penetration process. Chitosan gel seems to have less of an effect on the reduction of fluid penetration than calcium alginate when compared at the same concentration. As a result, the DTS value was reduced in nd-FSCPC using chitosan relative to that in nd-FSCPC using sodium alginate. Neither gels inhibited the process of fluid penetration into the cement paste completely. nd-FSCPC using chitosan showed inferior mechanical properties to that using sodium alginate. However, the results of this study indicate the possibility of producing nd-FSCPC with better mechanical properties; some additive capable of forming a water-insoluble gel to reduce fluid penetration more efficiently may be found in the near future. For better mechanical properties, smaller amounts of additive seem to be desirable. Ishikawa *et al.* reported that the DTS value of nd-FSCPC free from fluid exposure but maintained at 37 °C and 100% humidity decreased with the amount of sodium alginate included [1]. Although one of the requirements for additives is that they should not decrease the mechanical properties of set cement (requirement 4), a decrease in mechanical strength is natural since neither sodium alginate nor chitosan are desirable for HAP formation or interlocking of formed HAP crystals [8]. Fig. 4 also shows that larger amounts of chitosan were not preferable for higher mechanical strength. The DTS value of nd-FSCPC containing 1.5% chitosan was lower than that containing 0.5% chitosan ($p < 0.01$) . Chitosan thus has opposing effects on mechanical strength; inclusion of chitosan reduced the fluid penetration process as described above, however, chitosan also decreased the mechanical strength of set cement by interfering with the HAP interlocking reaction. Ideally, nd-FSCPC should show a DTS value of 10–12 MPa without fluid exposure, if there was an ideal additive capable of inhibiting fluid penetration completely in very small amounts.

When cements are used for load-bearing structures, higher mechanical strength is required. At present, however, both nd-FSCPCs using sodium alginate or chitosan show DTS values which are too low when the cement is fully exposed to fluid. An alternative way to increase the mechanical properties may be to accelerate the process of CPC replacement with bone [8]. Chitosan is known to have a pharmacological osteoconductive effect which favours the replacement process with bone [17–25]. Evaluation of the pharmacological effects of nd-FSCPC using chitosan was not within the scope of this investigation. However, the evaluation of osteoconductivity or bone-replacement behaviour of this cement will be the next step.

In conclusion, this study evaluated the feasibility of clinical use of nd-FSCPC using chitosan. Addition of chitosan, which forms a water-insoluble gel similarly to sodium alginate, into the liquid phase of FSCPC led to formation nd-FSCPC. Although, the degree of reduction of fluid penetration was poorer in cement containing chitosan than sodium alginate, thus showing poorer mechanical properties, the pharmacological effects of chitosan may lead to improved osteoconductivity of nd-FSCPC using chitosan. We recommend 0.5% chitosan for production of nd-FSCPC since decay of the cement paste was perfectly inhibited at this concentration and showed the highest DTS value. *In vivo* evaluation of nd-FSCPC using chitosan is awaited based on this initial *in vitro* study.

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