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Controllable release of salmon-calcitonin in injectable calcium phosphate cement modified by chitosan oligosaccharide and collagen polypeptide

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Abstract The aim of this research is to study the effect of the controlled releasing character of the salmon calcitonin (S-CT) loaded injectable calcium phosphate cement (CPC) modified by adding organic phase, chitosan oligosaccharide (CO) and collagen polypeptide (CP). The uniform design was used to determine the basic formulation with suitable injectable time for clinical application, and then the changes of the physical characters, the controlled releasing character of the modified CPC along with the ratio of the organic phase were also evaluated in vitro. The surface morphous of the modified CPC been implanted in the abdominal cavity or soaked into the serum of rat was also observed by scanning electron microscope (SEM). The result shows that a suitable formulation of modified CPC could be got, and the injectable time is 12 min, the compressive strength is 12 MPa, and the final setting time is 40 min. Comparing with the CPC without organic phase, the releasing rate of S-CT would increase along with the increase of the organic phase after 7th day. Therefore, a novel S-CT loaded bioactive injectable CPC for treating osteoporosis induced bone defect was obtained, and the release of the containing S-CT was controlled easily through adjusting the ratio of CO and CP.

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Introduction

Chow has successfully prepared the self-setting calcium phosphate cement (CPC) in 1986 [1]. It has been widely concerned because of its favourable characters, such as self-setting, good biocompatibility, optional moulding, and so on. On the base, injectable CPC (i-CPC) has been developed for an uninvasive surgery [2–9]. The plasticity is the most important character of CPC or i-CPC, which means that by regulating the prescription, the related parameters, such as the injectable time, porosity, strength and the degradation speed etc., can be adjusted for the different clinical applications. Besides, through the simple physical mixture, it can also be an excellent carrier for the growth factor, medicine or gene.

Because of its special properties, a $Ca_4(PO_4)_2O$ (TTCP) based injectable CPC system has been comfirmed through the Uniform Design in our laboratory, which has better injectability, setting time and strength. At the same time, salmon calcitonin (S-CT), a kind of drug for osteoporosis, is loaded in it for the uninvasive therapy of bone defect caused by osteoporosis [10].

For osteoporosis, it is well known that the proliferation capability of osteoblast is very low while the activity of osteoclast is enhanced, so the bone mass rapidly lost, and the bone is easily to be damnified. To cure such bone defect caused by osteoporosis, the local application of drug is an effective way to promote the differentiation and proliferation of the osteoblast and also to restrain osteoclast from continuing destroying the bone tissue, thus to improve the bone restoration rate and stability [11, 12].

Calcitonin (CT) is an effective drug and its mechanism is regarded as the first restrainer to cure the osteoporosis. However, some researches showed that the mechanism of calcitonin is not only to restrain the osteoclast, but it also

accelerate the proliferation and the differentiation of the osteoblast [13–15]. S-CT is a kind of polypeptide hormone, consisting of 32 amino acids, which activiate 30–40 times higher than the human calcitonin (H-CT). Clinical CT is usually used through intramuscular injection, which is not only cumbersome but also leads to toxic side effects because of the fluctuation of blood drug level. Therefore, we compounded S-CT with injectable CPC and resulted a kind of composite material with capability of releasing S-CT for treating the bone defect caused by the osteoporosis.

However, in the previous releasing experiment in vitro, we discovered the releasing speed markedly decreased two weeks later. It might due to the result of slow degradation of hydroxyapatite (HA), which is the final product of CPC. Therefore, in order to improve the releasing character and also other properties of the CPC system to suit the complex clinical application, we try to add some organic substances with better bioactivity and water-solubility in the original CPC system. By doing this, a system with better releasing character and physical properties is expected.

Chitosan oligosaccharide (CO) is derived from chitosan with enzyme hydrolysis, and is formed by several (2–10) β-1,4-glycoside linked amino glucose, which has perfect water-solubility, anticancer and antibacterial bioactivity [2, 16–20]. Collagen polypeptide (CP) is made by enzyme hydrolysis, it also has the perfect water-solubility and biocompatibility [21]. In this study, these two substances were added into the original CPC system, and the performance were investigated.

Materials and methods

Materials

CO (MingRang Bio. Chengdu, China) and CP (Haidebei Halobios. Jinan, China), prepared by enzymatic hydrolysis, both have the molecular weight of about 3000. $CaHPO_4 \cdot 2H_2O$ (DCPD, AR, Chongqing Che. Chongqing, China), hydroxyl apatite (HA, Guojia Bio. Chengdu, China) and $Ca_4(PO_4)_2O$ (TTCP), which were synthesized in our lab [22], were crushed by jet mill (model-STJ, Yixing, China) for getting the suitable granularity. Other reagents used in the experiment: S-CT (Sigma,USA); $CaCO_3$, $NaHCO_3$, citric acid (AR, Chongqing Che. Chongqing, China); 4-methylamine, acetonitrile, H_3PO_4 (HPLC, Shanghai Che. Shanghai, China).

Physical properties of the CO/CP/CPC composite

The clinical application will be strongly influenced by the injectablility, and the suitable injectable time is about 12 min. According to this clinical requirement, we can determine the basic formula of the composite through the method of uniform design. By considering the content of CO and CP in the solid phase as two variables, the Uniform Design 1.0 (software for uniform design, China) was used to get a factor 2 (CO and CP) lever 7 (1, 2, 3, 4, 6, 8, 10%) table (Table 1). The uniform design is an experiment design method introduced by Fang in 1980 which is to aim directly at the experiment with multiple levels and multiple factors [23]. Its characteristic is significantly reducing the number of tests especially for formula optimization. The Uniform Design 1.0 is based on the uniform design method, and offers the design table and the data statistics. Table 2 showed the main formulation of the samples been used in the experiments. The percentage of CO and CP in Table 2 corresponded to Table 1, for example, the percentage of CO in sample 1 corresponded to the level 1 and factor 1 in Table 1, and the percentage of CP in sample 1 corresponded to the level 5 and factor 2. In each sample, the percentages of CO and CP are variables, and the other components are dependent variables. After mixing of the solid phase and liquid phase with the ratio of 3:1 at 20 °C, the mixture was extruded from a 5-mL syringe equipped with needle (0.8 mm internal diameter, 12.34 mm in length). The reaction force on the syringe piston was tested by the Universal Testing Machine (WDW-50, China) with a displacement of 0.2 mm/s, and the injectable time was defined as the time when the reaction fore exceeded 20 N [24, 25]. Multiple linear regression of above indexes was made to study the effect of CO and CP on injectable time, and an optimized formulation with 12 min injectable time was determined. Based on this formula, the Table 3 was designed to study the changes of the setting time and compressive strength along with the ratio of organic phase. A batch of the mixture was injected into the sample mold of the Vicat apparatus at 37 °C, and then the initial and final setting times (I-time and F-time) were determined by Vicat needle test according to ASTM C191(the diameter of

Table 1 Uniform design table (2 factors, 7 levels)

Level	1(1%)	2(2%)	3(3%)	4(4%)	5(6%)	6(8%)	7(10%)
Factor 1 (CO)	1	2	3	4	5	6	7
Factor 2 (CP)	5	2	7	4	1	6	3

Table 2 Formulation of samples for the test of injectable time

Sample number	1	2	3	4	5	6	7
CO (wt.%)	1	2	3	4	6	8	10
CP (wt.%)	6	2	10	4	1	8	3
TTCP (wt.%)	48	49	44	47	48	43	44
DCPD (wt. %)	22	23	21	22	22	20	21
HA (wt.%)	12	13	12	12	12	11	12
Others (wt.%)	11	11	10	11	11	10	10

Table 3 Formulation of samples for studying the effect of different ratio of organic phase on physical properties

Sample number	1	2	3	4	5	6	7
Ratio of organic phase (wt.%)	4	7	10	13	15	17	20

needle is 1 mm; I-time is defined as the time when the needle subsides 25 mm in the sample; F-time is defined as the time when the needle can not make pressure mark on the surface of the sample). Another batch of mixture was injected in the cylindrical mold (6 mm internal diameter, 12 mm height) at 37 °C for 24 h after stripping for testing the compressive strength through the Universal Testing Machine (WDW-50, China) with cross-head speed of 0.5 mm/min.

The samples, contained different ratio (0, 5, 10, 15 wt%) of organic phase, were prepared for this test, and the S-CT concentration was 1,000 IU/L in liquid phase. After final setting, the samples were separately put into simulate body fluid (SBF) under a constant temperature of 37 °C. The lixivium on 1, 2, 3, 5, 7, 10, 20, 30 and 60 days was collected to determine the S-CT concentration by High Performance Liquid Chromatography (HPLC, LC-2010C, Japan). The test condition for HPLC was $ODSC_{18}$ (Dikma, USA), 220 nm, 40 °C, mobile phase (A:0.181% 4-methylamine:acetonitrile = 100:150; B:0.181% 4-methylamine:acetonitrile = 450:150, pH ~ 3.0 adjusted by H_3PO_4, A:B = 50:50).

Four kinds of samples (a: CO10%, CPC90%; b: CP10%, CPC90%; c: CO6.25%, CP3.75%, CPC90%; d: CPC100%) were prepared by mold (6 mm internal diameter, 12 mm height), and sterilized by ethylene oxide. One share of the samples was placed in diffusion chambers, and then was implanted into the rat's abdominal cavity. The other share was put into rat serum sterilized by 0.22 m filter membrane, and the serum was renewed each day under a constant temperature of 37 °C. All these samples including a share of control without any disposal were observed by SEM (JSM-5900LV, Japan) 10 days later.

Results and discussion

Physical properties of the CO/CP/CPC composite

The results of uniform design experiment were showed in Table 4. Because the suitable injectable time for clinical application was about 12 min, a basic optimal formula, the ratio of CO and CP was 3:5 and their total weight was 10% in the whole solid component, was determined by the multiple linear regression equation. When the ratio of organic phase was changed, the physical properties of composite would accordingly change, and the results were showed in Table 5. At the ratio mentioned above, the compressive strength would be 12 MPa, and the final-setting time would be 40 min. If the total weight of CO and CP exceeded 15%, the compressive strength of the material would decrease rapidly and the final-setting time would delay. If the ratio exceeded 10% but was lower than 15%, the compressive strength would keep increasing and the final-setting time would delay to 60 min or more. So the formula mentioned above could be the final formula with suitable physical characters for subsequent experiments.

It is difficult to describe the relationship between the physical properties and the variable with a precise and predictable constitutive equation due to the complexity of the impacting factors. So only some qualitative analysis could be made. As a biomacromolecule with low molecular weight, CO has amidogen and hydroxyl groups on its side chains [26]. Likewise, CP has two same groups, and carboxyl group as well [27]. When CO and CP were added into CPC, the hydrogen bond and ionic one would form between CO, CP, and CPC [28], thereby the compressive strength would be increased and the injectable time and setting time would be shortened. On the other side, the

Table 4 Results of uniform design experiment (n = 6)

Sample number	1	2	3	4	5	6	7
Injectable time (min)	10.2 ± 1.3	13.5 ± 2.1	8.4 ± 1.8	12.4 ± 2.6	13.9 ± 2.7	7.2 ± 1.1	8.5 ± 1.4

Data expressed as mean ± standard deviation

Table 5 Effect of different ratio of CO and CP on physical characters of the composite (n = 6)

Sample number	1	2	3	4	5	6	7
Initial setting time (min)	9.4 ± 1.1	10.2 ± 2.2	15.3 ± 2.0	16.8 ± 1.5	16.9 ± 2.4	19.8 ± 1.7	25.6 ± 2.8
Final setting time (min)	34.6 ± 2.9	35.8 ± 2.1	40.2 ± 2.7	55.8 ± 3.2	62.1 ± 2.1	65.1 ± 4.2	70.3 ± 4.6
Compressive strength (MPa)	6.2 ± 1.4	9.5 ± 1.6	12.3 ± 1.1	13.8 ± 1.2	14.1 ± 2.3	10.6 ± 2.1	6.3 ± 1.0
Injectable time (min)	11.0 ± 1.3	9.8 ± 0.9	11.8 ± 1.5	7.5 ± 0.8	6.8 ± 0.9	15.3 ± 1.8	20.5 ± 3.1

Data expressed as mean ± standard deviation

addition of CO and CP would block the hydration of CPC and inhibit the formation of HA crystal, thereby the compressive strength would be decreased and the injectable time and setting time would be prolonged. This showed that the effect of adding CO and CP on physical characters of CPC is bidirectional and complex, so it was difficult to make quantitative analysis. For this reason, the uniform design was used to optimize the compounding for clinical application. Furthermore, both CO and CP have excellent water-solubility [26, 27], the dissolution of them would lead to the formation of the new pores and the extension of the original pores, and this might offer the porous scaffold for bone growth.

In vitro releasing of S-CT in the CO/CP/CPC composite

Our previous study showed that S-CT stably released from the CPC without CO and CP in 10 days, and the curve of time$^{1/2}$—releasing amount was in accordance with the Hugichi equation [10]. However, the releasing amount markedly reduced thereafter, which might be resulted from the slow degradation of HA, the final product of CPC.

For CO/CP/CPC system, the results of releasing experiment were showed in Fig. 1. The releasing rate of the previous CPC in the first 5 days was 19%, which was slightly higher than the CO/CP/CPC composite. In 20 and

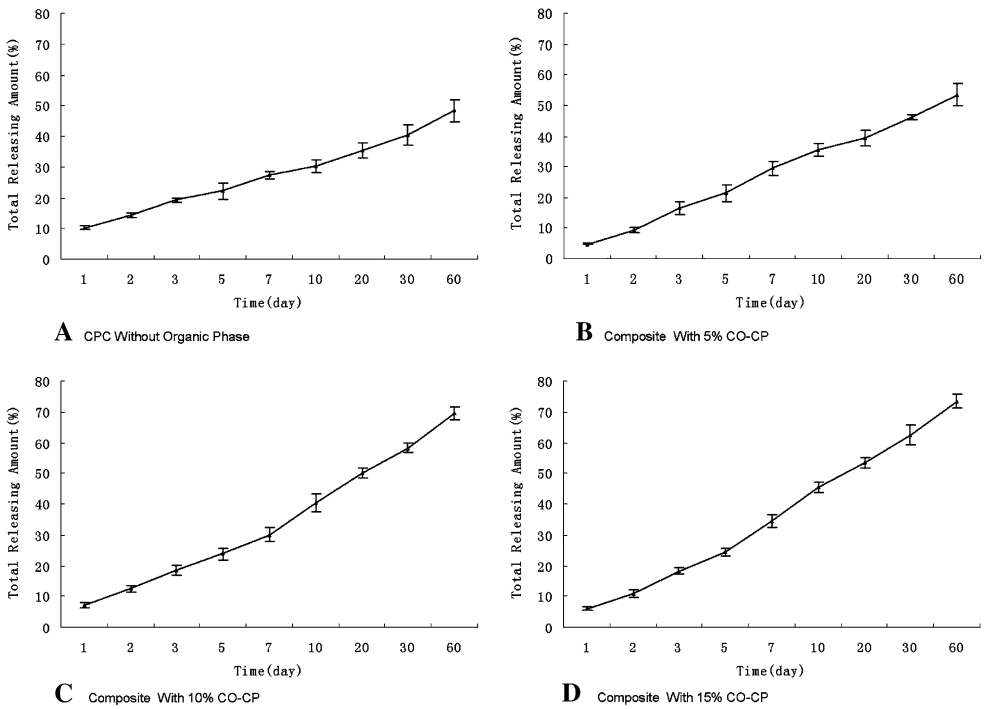

Fig. 1 In vitro releasing of S-CT in the CO/CP/CPC composite

60 days, the total releasing rates of the previous CPC were respectively 34% and 42%, but those of the CO/CP/CPC system with 5%, 10% and 15% of the organic phases were 46% and 52%, 55% and 62%, 58% and 69%. When the content of CO and CP was between 5% and 15%, the composite had a lower releasing amount in the first 5 days, as might be induced by the lower porosity. About 7 days later, due to the water-solubility of CO and CP [26, 27], the releasing rates increased with the increasing of the organic phases. As we know, the S-CT embedded into the interior, as well as those binding to the organic phases, would be released with the dissolution of CO and CP. Moreover, the new-formed transmission channels by that also accelerated the releasing of S-CT. According to that, the releasing rate of S-CT was effectively under the controlling of the adjustment of the proportion of organic phases in CPC. So the loading of some bioactive matters with various releasing demands for bone repair could be realized by this CO/CP/CPC system.

Effect of CO and CP on formation of bioactive layers on the surface of the CO/CP/CPC composite

Generally, both osteoinduction and osteaconduction could be promoted by formation of bone-like apatite on the material surface [29]. As a result, two methods of soak in vitro and implantation in vivo were taken to study the effect of biomineralization on CPC with different content

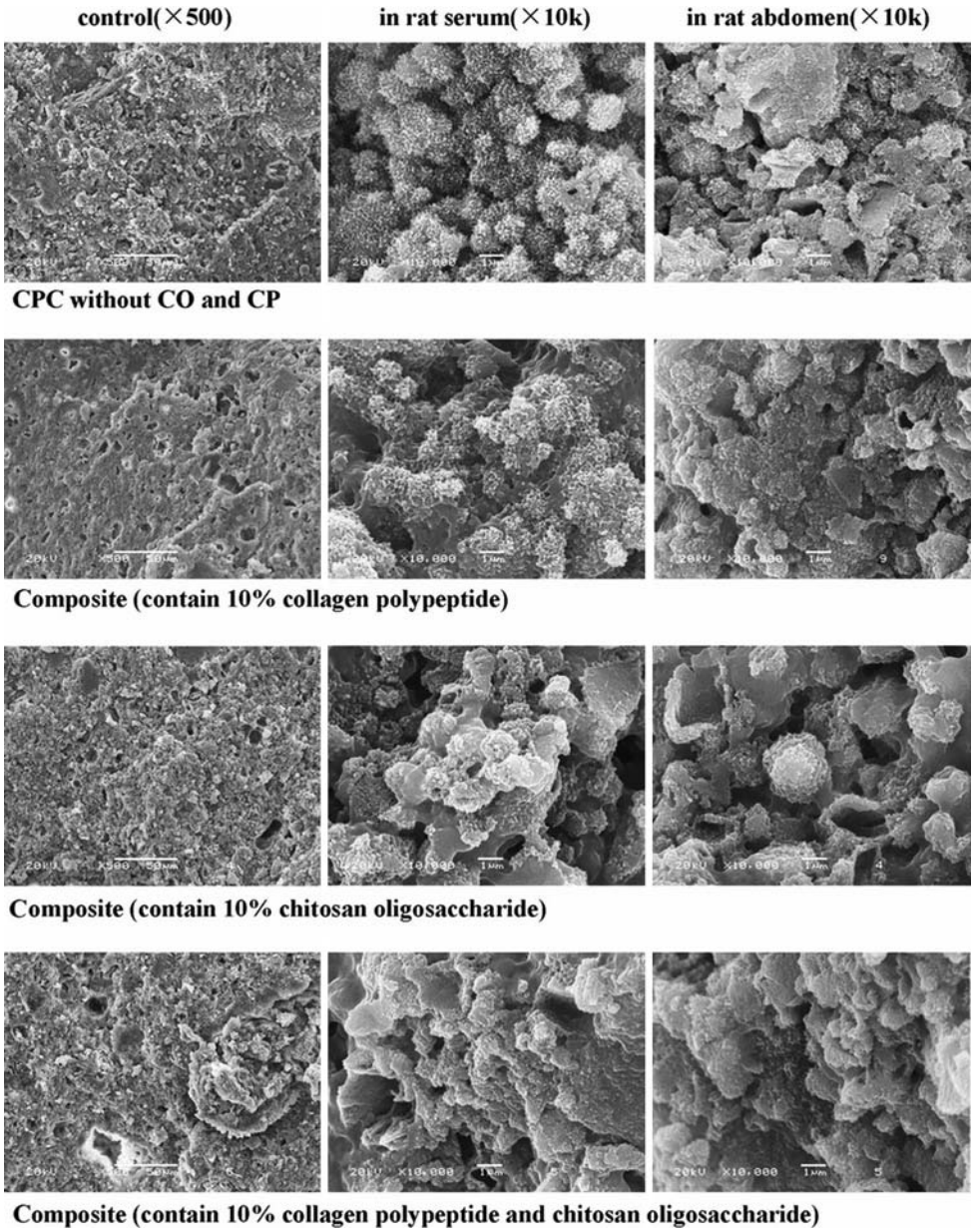

Fig. 2 Effect of CO and CP on forming of bioactive layers on the surface of the CO/CP/CPC composite

of CO and CP. As the optimal formulation whose organics content was 10% had been determined, in this experiment we compared the optimal formulation with sample contained 10% CP or 10% CO only, in order to learn the effect of CO and CP on biomineralization. In in vivo experiment, the samples were placed into diffusion chamber and then implanted into rat's abdominal cavity. By this way, the precipitation of inorganic salt in vivo would not be affected by the cells. Considering the obvious difference of abdomen fluid and tissue fluid in the composition, the static soaking method in rat serum was used. The results of SEM observations were showed in Fig. 2. Either in vivo or in vitro, the same kind of material had similar surface morphous. But for different kind of materials, the morphous was certainly different, and the crystal on the surface of CPC without CO and CP looked more confertim than all the samples which contained organics. These results suggest that the biomineralization more depends on the composition of the material, and the little soaking method. We surmise that this is because the fast dissolution of the organic phases might inhibit the aggradation and crystallization process. As for the effects of inhibition on the bioactivity of the materials, it is required to be proven through further studies.

Osteoporosis caused bone defect is different from normal mechanical defect, and the activity of osteoclast is markedly enhanced while that of osteoblast is reduced [11, 12]. As a general strategy of clinical treatment, we should inhibit the activity of osteoclast while promote that of osteoblast. In many papers, the bone related growth factors, such as BMP, were added into materials to improve the formation of new bone [30], but the adding of inhibitor was more important for osteoporosis. S-CT has both functions and so as to be taken in this research [10]. However, the inhibition of osteoclast perhaps may take negative effect on the degradation and absorption of the implant. Similarly, unfit addition of the organic phases has some negative influences on the physical properties and the biomineralization etc. Because of the existence of such contradiction, it is important to explore a balance point in these complicated factors and this is the key factor that a widely applicable CPC system could be constructed in our research.

Conclusion

Addition of chitosan oligosaccharide and collagen polypeptide can change the physical and releasing properties of S-CT loaded injectable CPC evidently. It is possible to suit the clinical application by opitimzing the amount of additives in CPC. Owing to its high plasticity in clinical application and predictable bioactivity in vivo, this kind of S-CT loaded composite material might be a preferable choice in individual and uninvasion treatment of osteoporosis caused bone defect.

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