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The optimum zinc content in set calcium phosphate cement for promoting bone formation in vivo

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Abstract

The final aim of our study is to develop a novel calcium phosphate cement based on zinc-containing α -tricalcium phosphate (α ZnTCP) and evaluate its potential as bonegraft material *in vivo*. In the present study, *in vivo* efficacy of zinc in hardened bodies of α ZnTCP was explored. The hardened bodies prepared from α ZnTCP with zinc content of 0.00, 0.04, 0.08, 0.11 and 0.19 wt % were prepared by mixing pure α TCP or α ZnTCP powder with 12 wt% sodium succinate solution at a solid-to-liquid ratio of 2.0. Due to the release of zinc ions into the physiological salt solution during curing, the zinc content in the hardened bodies was calculated to be 0.00, 0.03, 0.06, 0.10 and 0.18 wt%, respectively. The hardened bodies were implanted in the femora and tibia of white rabbits for 4 weeks. Histological and histomorphometric evaluation showed that the hardened body containing 0.03 wt% zinc, significantly promoted more new bone formation without evoking adverse tissue reactions than that without zinc. The hardened bodies containing 0.06 and 0.10 wt% zinc also resulted in the increase in numbers of active osteoblasts surrounding the new bone but caused inflammation at the implant sites. Results of this study indicate that the hardened body prepared with α ZnTCP is superior to that prepared with α TCP in promoting new bone formation due to the release of zinc ions. This study also indicates that the optimum amount of zinc in the hardened body is about 0.03 wt % to avoid inflammatory reaction.

Keywords

zinc; α -tricalcium phosphate; hardened body; bone cement; bone formation; histological and histomorphometric evaluation

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Introduction

Calcium phosphate cements (CPC) are clinically valuable due to their biocompatibility, ease of handling, moldability, injectability, ability to assume the shape and completely fill the bone defects, and ability to set or harden at body temperature. Calcium phosphate cements are classified into two basic systems: the tetracalcium phosphate (TTCP) and dicalcium phosphate anhydride (DCPA) system developed by Brown and Chow [1] and the α -tricalcium phosphate (α TCP) system developed by Monma [2]. α TCP cement has received much attention[3–7] owing to its unique characteristic of self-hydrolysis to form a hardened body. The hydrolysis of α TCP involves the dissolution of α TCP and the formation of calcium-deficient apatite (CDA) [8,9].

The zinc ion is involved in many metallo-enzymes and proteins including alkaline phosphatase (ALP) [10,11]. Zinc content in human bone (0.0126 ~ 0.0217 wt%), accounts for 28% of total body zinc, with 0.0030 wt% zinc in tissues and 12–17 Zn μM in plasma[12–15]. Zinc has a positive effect on factors of bone metabolism, such as growth hormone (GH) or insulin-like growth factor 1 (IGF-1) [16,17]. Zinc is an essential trace element for promoting osteoblast cell proliferation and differentiation [18,19], and inhibiting osteoclastic (bone resorbing) activity *in vitro* [20–22]. Zinc supplementation induces an increase in bone strength in growing rats[16]. And zinc supplementation at super physiological doses in healthy male humans increased total ALP activity, bone specific ALP activity and bone mass [23]. On the other hand, zinc deficiency resulted in a significant decrease in cortical and trabecular bone mineral density and in bone area in growing rats [24]. Zinc-deficiency is a risk factor of osteoporosis in humans [25,26].

Oral administration involves the process of absorption that is considered to be dose-dependent carrier-mediated process, and the efficiency of zinc absorption is inversely related to the amount of zinc in the diet [27]. In addition to the application of zinc as supplements, zinc incorporated in calcium phosphates (e.g., in tricalcium phosphate [28–31], apatite [30,32], organoapatite [33], bioactive glass [34]), ionomer cement [35] or in other ceramics (hardystonite, $\text{Ca}_2\text{ZnSi}_2\text{O}_7$) [36] have been investigated as potential materials to stimulate bone formation and inhibit bone resorption with promising results. However, it should be noted that elevated levels of zinc cause cytotoxicity. For example, Bioglass 45S containing $\geq 5\%$ (w/w) zinc is cytotoxic to human MG-63 cells and causes cell damage via oxidative stress [34]. In the case of βZnTCP ceramics, zinc content higher than 1.20 wt %, causes cytotoxicity to osteoblastic (MC3T3-E1) cells [28]. In the case of αZnTCP powder, when the zinc content is lower than 0.11 wt %, the *in vitro* cytocompatibility is at the same level as that of pure αTCP powder[37].

On the basis of above background, it is expected that the hardened bodies containing zinc at the optimum level based on αTCP cement system shows greater bone formation than that without zinc does without evoking adverse effects. The purpose of the present study was to explore the *in vivo* efficacy of the hardened bodies prepared from αZnTCP as a bonegraft material compared to that prepared from αTCP and to establish the optimum zinc content in the αZnTCP . In this study, the hardened bodies prepared from αZnTCP and from αTCP were implanted in rabbits and their efficacy in promoting bone formation was evaluated by histologic and histomorphometric measurements.

Materials and methods

Preparation of α TCP and α ZnTCP powders

The powders containing 0.00, 0.04, 0.08, 0.11 and 0.19 wt % zinc were designated α TCP, α ZnTCP-0.04, α ZnTCP-0.08, α ZnTCP-0.11 and α ZnTCP-0.19, respectively. α ZnTCP-0.11 or α ZnTCP-0.19 powders were prepared by heating β ZnTCP powder at 1400 °C for 5 hours. β ZnTCP powder containing 0.11 or 0.19 wt% zinc was fabricated by the solid state reaction between pure β TCP powder (Advance, Japan) and β ZnTCP powder containing 6.17 wt% zinc (10 mol%) at 850°C, sieved to smaller than 75 μ m, and ground for 20 minutes using an automatic mortar. The pure β TCP powder was also heated in the same manner as described above to obtain α TCP powder. Then, α TCP and α ZnTCP-0.11 powders were mixed to prepare α ZnTCP-0.04 and α ZnTCP-0.08 powders.

The compositions and particle sizes of all the powders used are summarized in Table 1.

Preparation of hardened bodies

α TCP or α ZnTCP powder was mixed well with 12 wt% sodium succinate aqueous solution at the solid-to-liquid ratio of 2.0 (g/ml). The mixture was loaded into a plastic cylinder mold and hardened at 37°C in 100 % humidity for 3 hours, followed by immersion in physiological saline at 37°C for 7 days for curing. Cylindrical hardened bodies with the final dimensions of 5.0 $\pm$ 0.5 mm in length and 3.5 $\pm$ 0.3 mm in diameter were prepared from mixtures of α TCP and α ZnTCP powders for animal study. These hardened bodies, designated Zn00, Zn04, Zn08, Zn11 and Zn19 (according to their zinc contents), were dried at room temperature and sterilized with ethylene dioxide gas before implantation in animals.

Characterization of powders and hardened bodies

Particle size distributions of α TCP and α ZnTCP powders were measured after dispersion in ethanol by ultrasonic radiation for 20 s using an Inspection System CIS-1 (GALAI, Israel) laser light scattering particle size analyzer. The phases present in the hardened bodies were examined by X-ray diffraction (Rigaku RINT-2500, Japan). The contents of hydroxyapatite (HAP) phase in the hardened bodies were determined from the peak intensity ratio of HAP (211) to α TCP (170) diffraction peaks. The compressive strength of the hardened bodies was measured at a crosshead speed of 0.1mm/min using a universal testing machine (Model Tensilon, Instron Co. Ltd, USA). The compressive strengths were examined for hardened bodies cured for 1, 4 and 7 days without drying. Differences in HAP content (n=3) and compressive strength (n=5) were analyzed using the unpaired *t* test. Fracture surfaces of hardened bodies were observed using a scanning electron microscope (SEM; Model SM-300, TOPCON Co. Japan)

Surgical procedures

Adult males (skeletally mature) New Zealand White rabbits (average weight, 3.0 kg) were used. The rabbits were anesthetized by intravenous injection of pentobarbital (40 mg/kg body weight). The rabbit's femoral great trochanter was penetrated with a drill bit 3.6 mm in diameter, using a strict aseptic technique. Then, the Zn00, Zn04, Zn08 or Zn11 implant was placed in the medullary cavity in a direction parallel to the long axis of the femur. Implants with the same zinc content were placed in the bilateral femora of the same rabbit. A total of 10 implants (two Zn00, two Zn04, two Zn08 and four Zn11 implants) were inserted into the femora of 5 rabbits. Postoperatively, each rabbit was allowed to move freely in its own cage, and was sacrificed after 4 weeks by an intravenous injection of excessive amount of pentobarbital.

Histomorphometric study was also carried out using adult, male, and skeletally mature Japan White rabbits around 3.0 kg in weight. After an intravenous injection of barbiturate (40 mg/kg body weight), small (10 mm) incisions were made on the skin in the medial proximal tibia using a strict aseptic technique. Then, the anteromedial cortex at proximal tibia was penetrated with a drill bit 3.6 mm in diameter perpendicularly to the long axis of the tibia. A Zn00 or Zn04 implant was inserted into the drilled hole and positioned such that one bottom surface of the hardened body was at the same level as the external surface of the tibia. A total of 12 implants were inserted in the tibiae of 6 rabbits. When the Zn00 implant was inserted into one tibia, the Zn04 implant was also inserted into the other tibia of the same rabbit. After the implantation, the skin was tied with two 3-0 non-resorbable sutures. Postoperatively, each rabbit was allowed to move freely in its own cage, and was sacrificed after 4 weeks.

The animal experiments were performed according to the guidelines of the Ethical Committee of the University of Tsukuba, the guidelines of the Ethical Committee of Tokyo Medical and Dental University, and the National Institute of Health guidelines for the care and use of laboratory animals (NIH Pub. No. 85-23 Rev. 1985).

Histological and histomorphometric studies

The scheme for the implantation and the new bone formation in the femoral specimens was shown in Fig. 1A. The femoral specimens were fixed in 4% neutral buffered formalin, decalcified with EDTA, embedded in paraffin and stained with hematoxylin and eosin, and sliced 5 μ m thick perpendicular to the long axis of the femoral bone for histological examination. The decalcified sections were examined using light microscopy (BX-51, Olympus Optical Co., Ltd., Tokyo, Japan); and images were captured using a high-sensitivity cooled CCD color camera (Olympus HC-300/OL, Japan).

The tibia specimens were fixed in 70% ethanol, stained with Villanueva bone stain, embedded in methylmethacrylate, sliced perpendicular to the long axis of the tibial bone, and ground to a thickness of 30 μ m for histomorphometric analysis. In two of six rabbits, two undecalcified histological sections were obtained from each implant, and one undecalcified histological section was obtained from each implant in the other four rabbits. Differences in the bone formation between the Zn00 ($n = 8$) and Zn04 ($n = 8$) were analyzed using the paired t test. In all cases, $p < 0.05$ was considered statistically significant. The undecalcified sections were observed with a stereo-zoom microscope (Olympus SZH, Japan); images were captured using a high-sensitivity cooled CCD color camera (Keyence VB-7010, Japan). The images of undecalcified sections were analyzed histomorphometrically with an image analysis software (Image Pro Plus 4.0 J) to determine the bone formation area (BFA), medullary cavity area (MCA), area of implant in the medullary cavity (AIMC) and bone apposition rate (BAR) in the intramedullary region, defined as follows (Fig. 1B, Table 2). The BFA (the sum of the new bone area S1, S2, S3 and S4) was defined as the area of new bone in the medullary cavity formed by the osteoconduction from the anteromedial cortex at proximal tibia where the drilled hole was made. New bone formed by the osteoconduction from anterolateral or posterior cortex was excluded from BFA to avoid a systematic error caused by the difference of whether the hardened body was in contact with the anterolateral or posterior cortex or not: Due to individual difference in tibia size, some hardened bodies were in contact with the anterolateral or posterior cortex, which led to osteoconduction from the contacting point, while other hardened bodies not. The boundary between newly formed bone and original mature cortical bone was defined by extrapolation of original endosteal surface line. The newly formed bone consisted mainly of osteoid, woven bone and partially mineralized bone, and consisted partially of mature bone, which was proved based on the color observed under normal light and fluorescence microscopes. The MCA was defined as the area surrounded

by the endosteal surface and the implant surface, thus excluding the area of implant and including the BFA. The AIMC was defined as the implant area excluding that in the cortical area. The BAR was defined as the interfacial length between the implant and the directly bonded bone per length of implant in the intramedullary region. The image capture and analysis were carried out by a single person in a blind manner.

Results

XRD patterns of α TCP and α ZnTCP-0.19 powder and the hardened bodies Zn00 and Zn19 cured for 7 days are shown in Fig. 2. The hardened bodies Zn19 showed the absence of α ZnTCP and the presence of low-crystalline apatite similar to that of porcine bone (in c of Fig. 2B). The phase evolution is same with α TCP (Fig. 2A). The HAP content in the hardened body and the compressive strength as a function of curing time of Zn00 and Zn19 hardened bodies are shown in Fig. 3. The amount of HAP in the hardened bodies showed no significant difference at all measuring points. The HAP content was 33–37 wt% after 3 hours and before curing in water at 37°C for both hardened bodies. After 1-day curing, the apatite contents increased to 88–90 wt% and almost all the α TCP phase was hydrolyzed to apatite after 4 days for both Zn00 and Zn19. There was no significant difference in compressive strength between hardened bodies Zn00 and Zn19 (Fig. 3). There was no appreciable difference in microstructure of the fracture surfaces between Zn00 and Zn19 after curing for 4 days as shown in SEM images (Fig. 4). Aggregates of flaky crystals of low-crystalline apatite with similar shape and size, formed by hydrolysis of α TCP or α ZnTCP powder, can be observed in both fracture surfaces. The above results confirm that the addition of 0.19 wt% zinc to α TCP has no adverse effect on setting properties of α TCP at 37°C.

The chemical compositions of hardened bodies Zn00, Zn04, Zn08, Zn11 and Zn19 are shown in Table 3. The zinc content in the hardened bodies Zn00, Zn04, Zn08, Zn11 and Zn19 was calculated to be 0.00, 0.03, 0.06, 0.10 and 0.18 wt%, respectively. The zinc contents of hardened bodies were slightly lower than those of the initial mixtures of α TCP and α ZnTCP-0.11 due to the release of zinc ions into the physiological salt solution during curing. Hardened bodies containing 0.03, 0.06 and 0.10 wt% zinc resulted in the increase in numbers of active osteoblasts and thus may stimulate active bone formation compared with that without zinc, but the hardened bodies containing 0.06 wt% zinc or higher caused an inflammatory tissue reaction. After 4 weeks of implantation, new bone formation was observed on the surfaces of all hardened bodies (Fig. 5). A large number of active osteoblasts with cuboidal morphology lined the newly formed bones surrounding the implanted hardened bodies in the cases of Zn04, Zn08 and Zn11. However, the number of active osteoblasts surrounding the new bone around Zn00 was apparently lower than those around Zn04, Zn08 and Zn11. Increasing in numbers of leukocytes and clots in the medullary cavity was used to judge the presence of inflammation. The formation of many clots and the presence of many leukocytes in the medullary cavity can be seen around hardened bodies Zn08 and Zn11, indicating presence of inflammation (Figs. 5E and 5F; Fig. 5G and 5H). On the other hand, no or only slight inflammation was observed around the hardened bodies Zn00 and Zn04 (Fig. 5A and 5B; Fig. 5C and 5D). The above results indicate that hardened bodies with zinc contents of 0.06 wt% or higher caused an inflammatory tissue reaction while those less than 0.06 wt% caused no inflammatory tissue reaction accompanying active bone formation.

The amount of bone formation around Zn04 was significantly higher than that around Zn00 without evoking inflammatory reaction. Based on the results of histological examination, hardened bodies Zn04 and Zn00 were used for the histomorphometric examination using undecalcified sections. Micrographs of undecalcified sections of hardened bodies Zn04 and

Zn00 implanted in rabbit proximal tibial metaphysis for 4 weeks are shown in Fig 6. New bone formed in the periphery of the drilled hole in continuity with the pre-existing bone and extended along the implant surface. The new bone bonded directly on the implant surface both in the intramedullary region and in the cortical region. The total area of new bone formed around Zn04 ($0.62 \pm 0.49 \text{ mm}^2$) was significantly higher than that around Zn00 ($0.31 \pm 0.29 \text{ mm}^2$) with $p = 0.02168$ (Fig. 7A). The area of new bone formation per area of implant in the medullary cavity for Zn04 ($4.30 \pm 3.54 \%$) was significantly higher than that for Zn00 ($2.07 \pm 1.80 \%$) with $p = 0.02453$ (Fig. 7B). The bone formation area per medullary cavity area for Zn04 ($1.89 \pm 1.40 \%$) was significantly higher than that for Zn00 ($1.03 \pm 0.95 \%$) with $p = 0.02432$ (Fig. 7C). However, the bone apposition rate (reflecting osteoconduction) was not significantly different between Zn04 and Zn00 (Fig. 7D).

Discussion

In general, α ZnTCP incorporating 0.19 wt% zinc or less has no adverse effect on the hydrolysis rate and the mechanical strength compared with pure α TCP, which shows a possibility of α ZnTCP for usage as a component of calcium phosphate cement instead of pure α TCP.

The optimum amount of zinc in the hardened body was about 0.03 wt%. Hardened bodies containing up to 0.10 wt% zinc were osteoconductive based on newly formed bone observed on the implant surface in the histological evaluation of decalcified sections (Fig. 5). However, hardened bodies containing 0.10 and 0.06 wt% zinc caused inflammatory reactions. On the contrary, the hardened body containing 0.03 wt% zinc caused no inflammation. Moreover, quantitative evaluation of the undecalcified sections showed that the hardened body containing 0.03 wt% zinc resulted in an increase in the amount of new bone formation compared with that without zinc (Fig. 7). Therefore, the optimum amount of zinc in the hardened body for use as calcium phosphate cement was about 0.03 wt%.

Both inflammatory reaction and increasing in numbers of active osteoblasts surrounding the new bone are the effects mediated by zinc since it is clearly evident that zinc content is the only difference among the hardened bodies used. It is clear that the hardened bodies implanted have the same apatite content, crystallinity, compressive strength, microstructure as shown in Fig. 2, 3 and 4 even though zinc has inhibitory effect on apatite crystallization, thus hardening. Therefore, effect of difference in physical properties of hardened bodies on biological reactions can be ignored.

We propose that the hardened bodies prepared from α ZnTCP can promote bone formation due to the release of zinc ions. Zinc has been shown to have stimulatory effects on bone formation around β ZnTCP and β ZnTCP/HAP ceramics [38]. The implantation experiments with rabbit femora showed that the optimum zinc content for β ZnTCP/HAP ceramics to promote bone formation was 0.316 wt% [38]. In the present study, the optimum zinc content the hardened bodies prepared from α ZnTCP was estimated to be 0.03 wt%. This means that the optimum zinc content is greatly reduced in the case of α ZnTCP without decreasing the effect of promoting bone formation compared with that of β ZnTCP ceramics and β ZnTCP/HAP ceramics. This phenomenon may be explained by the difference in the release rate of zinc ions caused by the difference in the solubility or dissolution rates of the carriers and the phase composition of the implant (hardened body). The hydrolysis product of α TCP or α ZnTCP is calcium-deficient apatite of low crystallinity; the rate of release of zinc ions from the hardened body will be greater compared with that from β ZnTCP or β ZnTCP/HAP which was sintered at high temperatures. Such characteristics enable the hardened bodies prepared from α ZnTCP to improve bone formation even though the zinc content is as low as 0.03 wt%.

Finally, setting behavior of the present α ZnTCP cement is yet to be fully improved. Setting time for the present α TCP and α ZnTCP incorporating 0.19 wt% zinc were 111 and 123 minutes, respectively, in average. Despite of the long setting time, we rather focused on clarifying the optimum zinc content in hardened body for promoting bone formation without evoking adverse effects. Since a 0.03 wt% zinc in hardened body was found to be optimum, the setting time can be improved by modifying phase and chemical composition of the powder and liquid within the range in which the final hardened body contains 0.03 wt% zinc. Further studies for improving setting behavior are required.

Conclusion

We demonstrated that the hardened body with a zinc content of 0.03 wt% obtained from zinc-containing α -tricalcium phosphate (α ZnTCP) stimulated greater bone formation compared with that obtained from α -tricalcium phosphate without zinc. The incorporation of zinc up to 0.19 wt% into α -tricalcium phosphate phase caused no change in the hydrolysis rate of α -tricalcium phosphate phase. Hardened bodies containing 0.03, 0.06 and 0.10 wt% zinc resulted in the increase in numbers of active osteoblasts, but the hardened bodies containing 0.06 wt% zinc or higher caused an inflammatory tissue reaction. The optimum zinc content in the hardened body is around 0.03 wt% for promoting bone formation without inflammation. Both the increase in numbers of active osteoblasts surrounding the new bone and the inflammation can be attributed to the release of zinc ions from the hardened body. Results of this study indicated that the hardened body containing small amount of zinc (0.03 wt%) prepared from α ZnTCP used as bonegraft material led to an increase in the amount of newly formed bone, even though osteoconduction remains unchanged.

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Fig. 1. Definition of histomorphometric parameters on a sectional surface. L_x : a length of an interface between an implant and a newly formed bone, L_{mx} : length of an implant from a bottom to an inner surface of a cortical bone, S_x : a area of newly formed bone.

Fig. 2.

X-ray diffraction patterns of (A): α TCP powder (a), the hardened bodies Zn00 cured for 7 days (b); (B): α ZnTCP-0.19 powder (a), the hardened bodies Zn19 cured for 7 days (b) and porcine bone (c).

Fig. 3. HAP content in the hardened body and compressive strength of the hardened bodies prepared from (A) pure α TCP and (B) α ZnTCP-0.19 after various curing times.

Fig. 4.
SEM images of fracture surfaces for the hardened bodies prepared from (A) α TCP and (B) α ZnTCP-0.19 powders cured for 4 days.

Fig. 5. Histological sections after implantation in rabbit femora for 4 weeks of the hardened bodies (A, B) Zn00, (C, D) Zn04, (E, F) Zn08 and (G, H) Zn11 containing 0.00, 0.04, 0.08 and 0.11 wt% Zn, respectively. Arrows and “H” indicate new bone and the hardened body, respectively.

Fig. 6. Undecalcified sections after implantation of the hardened bodies (A, B and C) Zn00, and (D, E and F) Zn04 in rabbit tibia for 4 weeks, prepared from α TCP and α ZnTCP-0.11 powders, respectively.

Fig. 7.

Results of histomorphometric evaluation: (A) Bone formation area (BFA); (B) BFA per area of implant in the medullary cavity (AIMC); (C) BFA per medullary cavity area (MCA); (D) bone apposition rate (BAR) for the hardened bodies Zn00 and Zn04 prepared from α TCP and α ZnTCP-0.11 powders, respectively. (* means statistically significant difference, $p < 0.05$)

Table 1
Chemical compositions, and mean and median particle size of α TCP, α ZnTCP-0.11 and α ZnTCP-0.19 powders.

Sample name	Chemical composition			Particle size	
	CaO wt %	ZnO wt %	P ₂ O ₅ wt %	Total wt %	Mean μ m
α TCP	54.15	<0.01	46.29	100.43	2.66 $\pm$ 1.68
α ZnTCP-0.11	54.43	0.14	46.06	100.62	3.44 $\pm$ 2.23
α ZnTCP-0.19	54.15	0.24	46.29	100.67	2.58 $\pm$ 1.62

Table 2

Definition of histomorphometric parameters expressed by symbols in Fig. 2.

Parameters	Calculation method
BFA	$S1+S2+S3+S4$
BFA per MCA	$(S1+S2+S3+S4)/MCA$
BFA per AIMC	$(S1+S2+S3+S4)/AIMC$
BAR	$(L1+L2+\dots+L7)/(Lm1+Lm2)$

Table 3

Chemical compositions of hardened bodies prepared from α TCP and α ZnTCP powders.

Sample	CaO (wt%)	ZnO (wt%)	P ₂ O ₅ (wt%)	Na ₂ O (wt%)	Total (wt%)
Zn00	50.63±0.42	0.00±0.00	43.88±0.28	0.49±0.01	95.00±0.70
Zn04	50.69±1.14	0.04±0.00	44.54±0.68	0.47±0.01	95.74±1.84
Zn08	49.84±0.91	0.07±0.00	43.80±0.81	0.47±0.01	94.19±1.73
Zn11	50.10±0.47	0.13±0.00	43.78±0.47	0.46±0.01	94.47±0.95
Zn19	49.85±0.53	0.22±0.00	45.03±0.45	0.63±0.00	95.73±0.98