FULL PAPER *Laboratory Animal Science*

Sustained Efficacy of Erythropoietin with a Hydroxyapatite Carrier Administered in Mice

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ABSTRACT. For chronic kidney disease patients with renal anemia, recombinant human erythropoietin (rHuEPO) is a very effective drug; however, the treatment regime is troublesome, requiring multiple administrations each week. In the present study, we examined the efficiency of hydroxyapatite (HAp) as a drug delivery carrier for the sustained release of erythropoietin (EPO) to reduce the frequency of administration. Spray-dried HAp microparticles, formed from zinc-containing HAp (Zn-HAp) and Zn-HAp calcined at 400°C, were used as carriers of EPO, and five Zn-HAp formulation samples incorporating EPO were prepared; no formulation, poly-L-lactic acid (PLA) formulation, zinc (Zn) formulation, Zn/PLA formulation, and calcined/Zn/PLA formulation. ICR mice were administered these samples or commercial rHuEPO (Epogin) as a control from dorsal neck subcutaneous, and hematological and histopathological analyses, including enzyme-linked immunosorbent assay for plasma EPO concentration, were performed. An increase in the blood EPO level was detected on days 3 and 8 post-administration. Peak hematopoiesis was delayed and higher hematological values were obtained on day 14 post-administration with no serious adverse reactions compared with the control. The Zn/PLA formulation sample was found to be most effective in reducing the initial peak while sustaining the delayed release of EPO. In conclusion, the Zn-HAp formulation samples were considered to be useful carriers for the sustained release of EPO, and the Zn/PLA formulation appears to be the most effective of five Zn-HAp formulation samples in sustaining EPO release.
KEY WORDS: anemia, erythropoietin, hydroxyapatite, sustained release.

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Anemia is a common complication of chronic kidney disease (CKD) [34, 36]. For CKD patients with renal anemia, recombinant human erythropoietin (rHuEPO) treatment has contributed to an improved quality of life, though the patients require treatment two or three times per week [9]. The development of a novel treatment method for anemia using a drug delivery system (DDS) has become very necessary.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$: HAp) is a biocompatible ceramic, widely used in the biomaterial field [14, 15, 27]. HAp particles have been examined for application in the sustained release of various therapeutic agents, such as antibiotics [1, 11, 26, 27], anticancer drugs [8, 35] and proteins [5, 6, 18, 19]. Nagao *et al.* has reported that HAp has the ability to absorb therapeutic agents without deactivation and shows regulated release as it biodegrades [22]. The biodegradation speed of HAp can be regulated by calcination temperature [19, 23, 31]. Spray-drying has been shown to be a good fabrication method for spherical porous HAp powder with a high specific surface area of more than 60 cm^3/g [23, 31]. Mizushima *et al.* [19] recently reported the possibility of the sustained release of proteins and lipophilic drugs using injectable HAp microparticles. The HAp microparticles calcined at 400°C were gradually degraded *in vivo* and the addition of human albumin and zinc ion during the HAp microparticle formulation prolonged the release of interferon alpha *in vivo*. Nagao *et al.* [22] have reported that HAp in which 5% calcium was replaced with zinc can absorb more therapeutic agents, and coating HAp particles with poly-L-lactic acid (PLA) extended the time to release of human growth hormone. However, the effects of these treatments on other bioactive proteins have not yet been reported.

In the present study, we examined the efficiency of HAp for the sustained release of erythropoietin (EPO). Zinc-containing HAp (Zn-HAp) produced using the spray-drying technique and Zn-HAp further calcined at 400°C were used as carriers for EPO, with five types of Zn-HAp formulation prepared after the incorporation of EPO; no formulation, PLA formulation, zinc (Zn) formulation, Zn/PLA formulation, and calcined/Zn/PLA formulation. These samples were examined to clarify which is the most effective formulation for the sustained release of EPO *in vivo*.

MATERIALS AND METHODS

Animals: Specific pathogen-free ICR male mice (5 weeks

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old) were purchased from Japan SLC, Inc. (Shizuoka, Japan), and used for the study at 6 weeks old. They were housed in plastic cages under standard laboratory conditions (temperature, 20–26°C; relative humidity, 30–80%; light-dark cycle, 12/12 hr) in the animal facility, Japan Food Research Laboratories. They were given a CRF-1 diet (γ-ray irradiated, Oriental Yeast Co., Ltd.) and tap water *ad libitum*. The study was conducted according to the ethical guidelines for laboratory animals and the standard operating procedure of Japan Food Research Laboratories. The experimental protocol was approved by the Committee for Animal Experiments Ethics of Japan Food Research Laboratories.

Hydroxyapatite carriers: Zn-HAp ($Ca_{9.5}Zn0.5(PO_4)_6(OH)_2$) was synthesized using a wet method at room temperature; the phosphate solution (0.15 M, 1*l*) including zinc chloride (6.8 g) was dropped into the calcium hydroxide suspension (0.25 M, 1*l*) at pH 8.0. The Zn-HAp suspension was spray-dried with a mini spray dryer (B-290, Büchi, Sweden) at an atomizing pressure of 0.56 MPa, outlet and inlet temperatures of 180°C and 70°C, respectively, and a gas flow rate of 30 m^3/hr to obtain spherical microparticles [29]. The microparticle(s) obtained were partially calcined at 400°C for 1 hr under a static atmosphere. EPO (Pestka Biomedical Laboratories., Product No.: 11965–9) was dissolved in 10 m*l* of 10% phosphate-buffered saline (PBS) into which 100 mg of Zn-HAp particles were immersed for 10 min at the ratio of 50 U of EPO to 1 mg of Zn-HAp. Solid-liquid separation was performed by centrifugation at 3,000 rpm for 10 min in all procedures. After adsorption of the EPO, the supernatant was discarded and 2 m*l* of zinc chloride solution (10 mg/m*l*, pH 5.5) was added prior to incubation for 3 min. The EPO/Zn-HAp suspensions with or without zinc chloride were centrifuged, washed with distilled water and ethanol, and dried at room temperature. Thirty mg of PLA was dissolved into 1 m*l* of methyl-chloride solvent into which 100 mg of EPO/Zn-HAp powder was added, and the solvent was completely evaporated at room temperature using an evaporator (Buchi, Sweden). Five different samples were obtained as shown in Fig. 1. These samples were characterized with a scanning electron microscope (JEOL JSM5600LV) and a particle size analyzer (Shimadzu, Salada-2000). Specific surface area was analyzed by the Brunauer-Emmett-Teller (BET) method using nitrogen gas. To determine the absorption capacities of EPO to Zn-HAp, we mixed HAp and EPO at different units and measured the amount of EPO that remained in the supernatant with enzyme-linked immunosorbent assay (Human erythropoietin ELISA kit, Stemcell Technologies Inc.). We found that at least 50 U of EPO was able to absorb to 1 mg of HAp (data not shown). However, we could not measure the biological units of EPO/Zn-HAp because the EPO absorbed into HAp was not released in saline similar to the earlier report [22]. In preliminary studies, when 500 U of EPO/Zn-HAp was injected subcutaneously, these mice showed the sustained efficacy of hemopoiesis without any side effects (data not shown). Thus, we used this dosage (500 U/body) to evaluate the therapeutic efficacy of sustained release of EPO in the present study. The dose of EPO administered to the mouse in two weeks was 8–40 fold of therapeutic human maintenance dose in the present study.

Hemanalysis, weight change of organs, and histopathological analysis: The above samples suspended in saline or Epogin (Chugai Pharmaceutical Co., Ltd.) as a control were subcutaneously administered to ICR mice (500 U/body). The carrier alone (Zn-HAp containing zinc and PLA with-

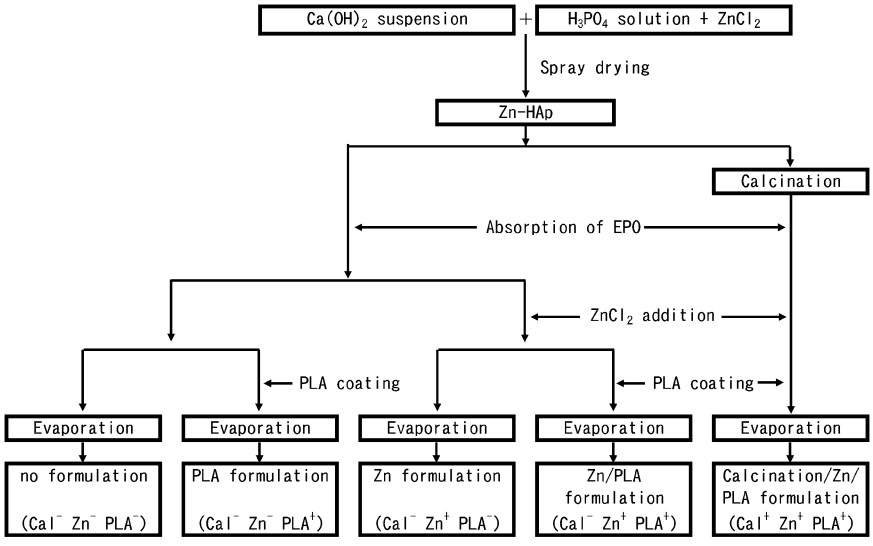

Fig. 1. Sample preparation protocol. Zn-HAp or Zn-HAp calcined at 400°C was used as carriers of EPO. The Zn-HAp formulation samples were prepared after adsorption of EPO. Abbreviations of the samples are shown in the bottom-most squares; $Cal^- Zn^- PLA^-$ (no formulation), $Cal^- Zn^- PLA^+$ (PLA formulation), $Cal^- Zn^+ PLA^-$ (Zn formulation), $Cal^- Zn^+ PLA^+$ (Zn/PLA formulation) and $Cal^+ Zn^+ PLA^+$ (Zn/PLA formulation after calcination).

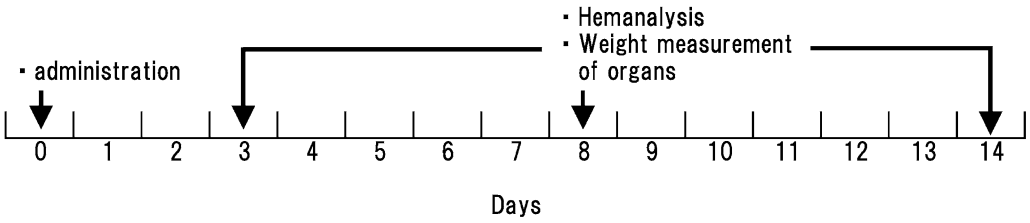

Fig. 2. Time course of this study. On days 1, 3, 8, and 14, hemanalysis (the number of red blood cells, hemoglobin concentrations, hematocrit values, and plasma EPO) were analyzed.

out EPO) or saline was each subcutaneously administered in the same way. Blood samples were collected from the post-cava under ether anesthesia on days 1, 3, 8, and 14 post-administration, and used for hematological analyses and the human erythropoietin ELISA kit for plasma EPO. The number of red blood cells, hemoglobin concentrations, and hematocrit values were analyzed with an automatic counter (KX-21NV; Sysmex Corporation) according to the instruction manual. For each hematological parameter, the sustaining rate, the ratio of the value on day 14 to that on day 1, was evaluated as the efficacy of hematopoiesis. Mice administered Cal^{-} Zn^{-} PLA^{-}, Cal^{-} Zn^{+} PLA^{-}, Cal^{-} Zn^{+} PLA^{+}, and Epogin, were sacrificed by excess ether inhalation and their enucleated liver, kidneys, and spleen were weighed. Each organ as well as the subcutaneous tissue from the administration site was fixed in 10% neutral-buffered formalin, and histopathological analyses were performed after hematoxylin and eosin staining.

Statistical analysis: Statistical analysis was performed between two groups, Zn-HAp formulation and Epogin groups, to detect the more effective formulation compared with aqueous EPO on the number of red blood cells, hemoglobin concentrations, hematocrit values, and organ weights using SPSS 11.0J for Windows [SPSS Japan Inc.]. Student's *t*-test was performed when the variance of the data from the samples and control was the same, whereas Welch test was performed when the variances differed. These values were represented as means ± SD and differences with $P<0.05$ were considered to be significant.

RESULTS

Zn-HAp microparticles and formulations: The porous spherical particles, shown in Fig. 3 (scanning electron microscopy image), had an average diameter of 5.2 μm and a particle size distribution of 1 to 20 μm. The particle size was not changed by the calcination at 400°C. The specific surface area of the spray-dried microparticles obtained using the BET method was 168 m^2/g, whereas that of the calcined microparticles was 104 m^2/g due to crystal growth during heating. The specific surface area of the PLA formulation was 63 m^2/g, which can be attributed to the limited incorporation of nitrogen gas into the microparticles. The amount of EPO adsorbed onto the various Zn-HAp microparticles was estimated by measuring the EPO concentration in the clear top layer of liquid remaining after the adsorption processes using an ELISA kit. The amount of EPO remaining in the liquid, however, was under the detection level of ELISA in each case, so that all of the EPO was considered to have been adsorbed onto the Zn-HAp surface.

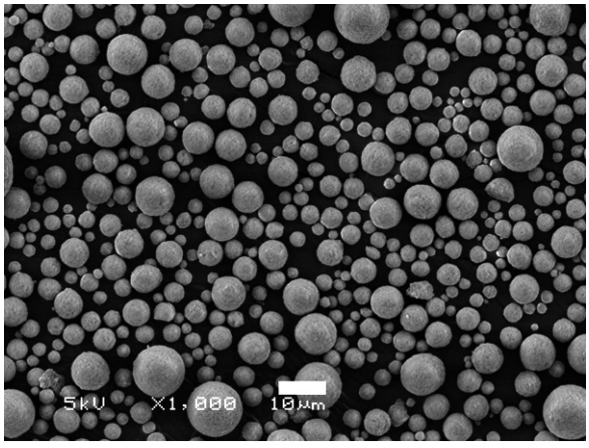

Fig. 3. Scanning electron microscopy image of spray-dried Zn-HAp microparticles. The average diameter of 100 particles was 5.2 μm.

Hematological data and plasma EPO levels: Table 1 and Fig. 4 show the changes in hematopoietic parameters, such as the number of red blood cells, hemoglobin concentration, and hematocrit value, in mice administered the five samples and Epogin as the control. In the data for the Epogin-administered mice, the peaks of nearly all hematopoietic parameters were observed on day 3 post-administration, whereas those of the mice administered each of the Zn-HAp samples were observed on day 8, suggesting that Zn-HAp delayed the effect of EPO on hematopoiesis (Fig. 4A, B, and C). This is supported by the data for plasma EPO levels (Fig. 4D). Thus, on day 3, plasma EPO level in mice administered Epogin was lower than those of mice administered each of the Zn-HAp samples except for Cal^{-} Zn^{-} PLA^{-}. Among the Zn-HAp samples, the efficacy of no formulation (Cal^{-} Zn^{-} PLA^{-}) sample was the lowest. Among the formulations samples tested in the present study, Zn and/or PLA formulations tended to be most effective in enhancing the efficacy of Zn-HAp. The sustaining rate was calculated to evaluate the efficacy of samples in sustaining hematopoiesis. As shown in Fig. 5, the Cal^{-} Zn^{+} PLA^{+} formulation was the most effective for all hematopoietic parameters, suggest-

Table 1. Hematological parameters post administration

Sample	Days	n	Red blood cells ($10^4/\mu l$)	Hemoglobin (g/d*l*)	Hematocrit (%)	Plasma EPO (mU/m*l*)
$Cal^- Zn^- PLA^-$	1	3	754 ± 32	12.4 ± 0.3	44.4 ± 1.2	4686.4 ± 1434.7
	3	3	795 ± 15	14.0 ± 0.4	50.9 ± 1.8*	0.8 ± 0.4
	8	3	887 ± 29	14.3 ± 0.1	51.2 ± 0.4*	ND
	14	2	800 ± 14	12.9 ± 0.4	45.9 ± 2.1	ND
$Cal^- Zn^+ PLA^-$	1	3	758 ± 49	12.8 ± 0.8	45.5 ± 2.7	7632.7 ± 948.0
	3	3	769 ± 37	13.7 ± 0.6	50.5 ± 3.1*	14.5 ± 15.5
	8	3	946 ± 64	15.4 ± 0.4*	56.7 ± 1.5*	1.3 ± 0.6
	14	3	914 ± 64*	14.8 ± 0.8*	53.9 ± 3.4*	ND
$Cal^- Zn^+ PLA^+$	1	2	721 ± 21	12.0 ± 0.1	41.8 ± 0.9*	33.0 ± 9.8
	3	6	812 ± 34	13.5 ± 0.5*	49.3 ± 1.8*	6.1 ± 4.3
	8	6	906 ± 67	15.2 ± 1.1	54.8 ± 4.1	1.1 ± 1.3
	14	6	916 ± 52*	14.7 ± 0.8*	52.9 ± 3.6*	ND
$Cal^+ Zn^+ PLA^+$	1	2	772 ± 33	12.3 ± 0.2	44.6 ± 1.7	42.0 ± 5.7
	3	3	798 ± 6	13.3 ± 0.4*	48.3 ± 1.0*	3.6 ± 1.4
	8	3	951 ± 76	15.8 ± 0.7*	57.0 ± 3.4	ND
	14	3	907 ± 73	14.8 ± 0.5*	52.2 ± 2.6*	ND
$Cal^- Zn^- PLA^+$	1	2	757 ± 20	12.2 ± 0.3	44.4 ± 0.8	37.1 ± 25.3
	3	3	816 ± 55	13.4 ± 0.6*	48.9 ± 3.2*	5.4 ± 7.4
	8	3	893 ± 45	14.6 ± 0.9	52.5 ± 3.4	ND
	14	3	927 ± 42*	14.4 ± 0.3*	51.8 ± 1.1*	ND
Epogin	1	3	721 ± 26	12.6 ± 0.1	45.3 ± 1.3	6977.3 ± 1236.7
	3	3	867 ± 68	14.9 ± 0.5	56.7 ± 2.1	1.4 ± 0.9
	8	3	878 ± 7	14.4 ± 0.3	52.9 ± 0.9	ND
	14	5	820 ± 26	13.5 ± 0.5	48.0 ± 1.8	ND
Carrier alone	1	3	752 ± 48	12.6 ± 1.0	44.3 ± 2.7	ND
	3	3	759 ± 31	12.6 ± 0.7*	45.3 ± 2.6*	ND
	8	3	778 ± 14*	13.4 ± 0.2*	46.2 ± 0.5*	ND
	14	3	819 ± 2	13.7 ± 0.3	47.8 ± 0.5	ND
Saline	1	3	737 ± 50	12.1 ± 0.4	43.8 ± 2.0	ND
	3	3	742 ± 38*	12.7 ± 0.3*	45.1 ± 1.1*	ND
	8	3	792 ± 9*	13.5 ± 0.3*	46.3 ± 0.7*	ND
	14	3	836 ± 27	13.8 ± 0.4	47.8 ± 1.1	ND

Values represent mean ± SD. ND: Not detected. *: $P<0.05$, versus Epogin.

ing that the Zn/PLA formulation is suitable for the sustained release of EPO *in vivo*.

Weight of organ(s) and histopathology: Table 2 shows the changes in weight of the liver, kidneys, and spleen after administration of the five samples and Epogin as a control. In all mice, the weight of the spleen increased until day 3. Although there was no statistical difference, the increase in spleen weight in the $Cal^- Zn^+ PLA^+$-administered mice was lower than that in mice administered with the other samples. No significant changes in the weight of the liver or kidneys were observed, for though significant differences were detected in the $Cal^- Zn^+ PLA^-$ group on day 14 and the $Cal^- Zn^- PLA^-$ group on days 1 and 8, these may be artificial differences due to accidental small standard error values. Finally, histopathological analyses of the dorsal neck subcutaneous tissue in mice administered the $Cal^- Zn^- PLA^-$, $Cal^- Zn^+ PLA^-$, and $Cal^- Zn^+ PLA^+$ microparticles were performed. All formulated particles were surrounded by fibrous membranes accompanied by fibroblasts and cellular infiltrations. Neither degeneration nor necrosis occurred in the tissue in which the Zn-HAp was injected (Fig. 6). Histopathological observation of the spleen showed an expansion of red pulp with extramedullary hematopoiesis, predominantly on day 3, in all mice administered the three samples or Epogin, which was abolished by day 14 (data not shown). No obvious abnormalities were found in the histological analyses of the liver and kidneys (data not shown).

DISCUSSION

The present study revealed that the Zn-HAp samples have the ability to sustain the release of EPO without serious adverse reactions and that the Zn/PLA formulation was the most effective in sustaining EPO release from Zn-HAp.

EPO is a 30–40 kDa glycoprotein hormone and is classified as a hematopoietic cytokine that regulates red blood cell production [12, 34]. Since the site of EPO production is the peritubular cells of the kidneys, in the majority of cases

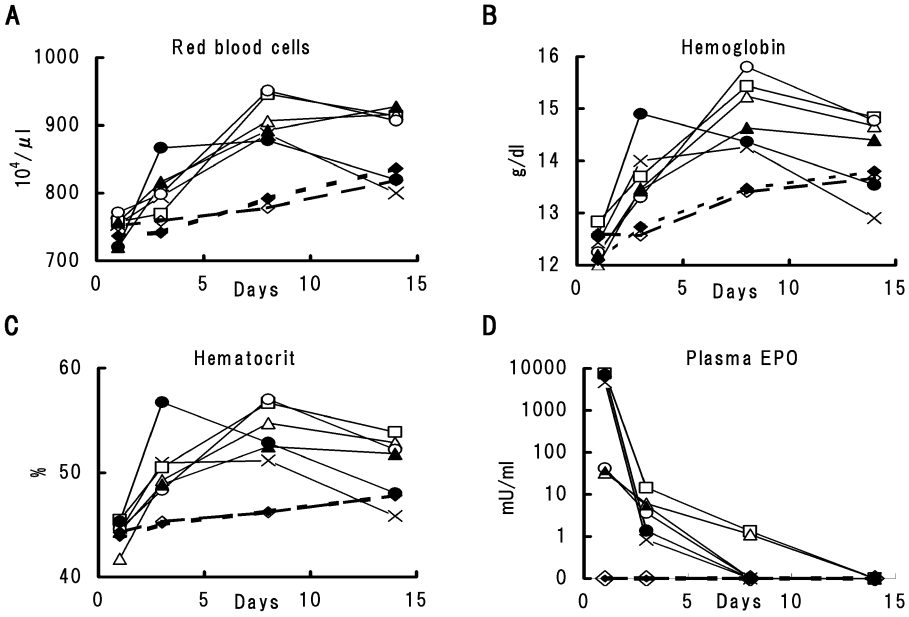

Fig. 4. Hematological parameters and plasma EPO levels. The number of red blood cells (A), hemoglobin concentration (B), hematocrit value (C), and plasma EPO level (D) was determined after administration. ×; Cal^{-} Zn^{-} PLA^{-}, □; Cal^{-} Zn^{+} PLA^{-}, △; Cal^{-} Zn^{+} PLA^{+}, ○; Cal^{+} Zn^{+} PLA^{+}, ▲; Cal^{-} Zn^{-} PLA^{+}, ●; Epogin, ◇; Carrier alone, ◆; Saline. In Cal^{-} Zn^{+} PLA^{-}, Cal^{-} Zn^{+} PLA^{+}, Cal^{+} Zn^{+} PLA^{+}, and Cal^{-} Zn^{-} PLA^{+}, a delay in peak hematopoiesis was observed and higher values sustained until day 14 post-administration compared with Epogin.

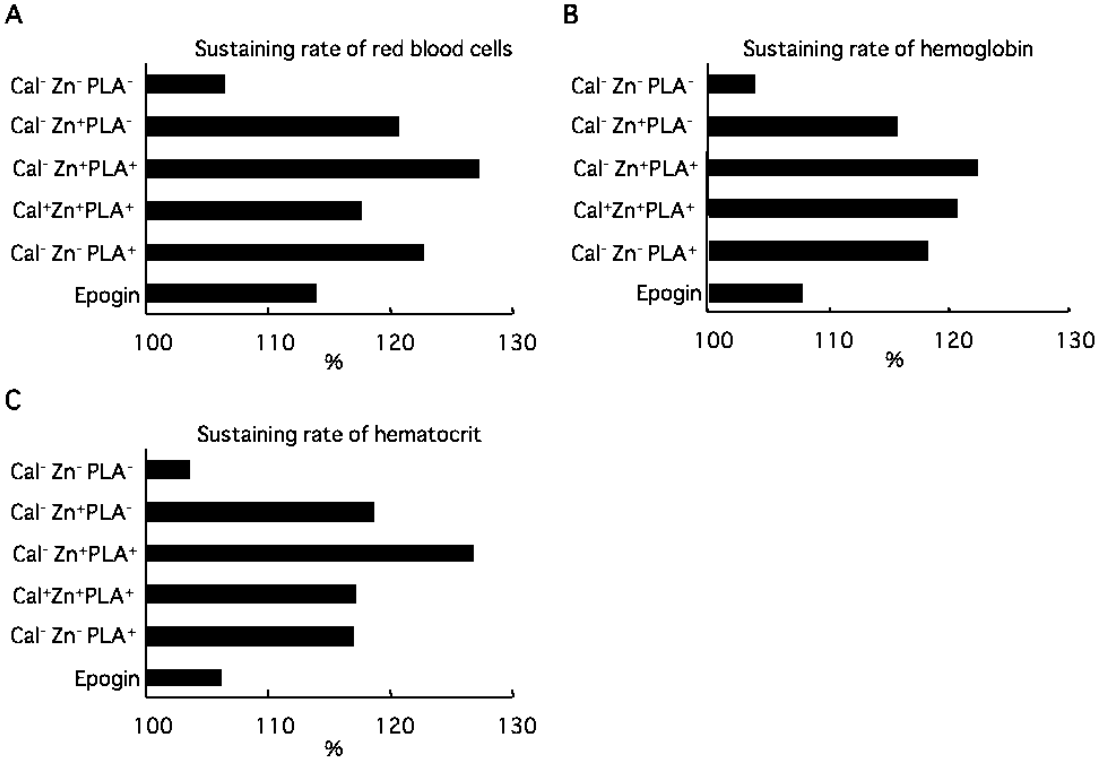

Fig. 5. Sustaining rate, the ratio of the value on day 14 to that on day 1, was calculated from the number of red blood cells (A), hemoglobin concentration (B), and hematocrit value (C). The rates of sustainability for Cal^{-} Zn^{+} PLA^{-}, Cal^{-} Zn^{+} PLA^{+}, Cal^{+} Zn^{+} PLA^{+}, and Cal^{-} Zn^{-} PLA^{+} were higher than that for Epogin.

Table 2. Weight of organ post administration

Sample	Days	n	Liver (mg)	Kidneys (mg)	Spleen (mg)
Cal^{-} Zn^{-} PLA^{-}	1	3	2267 ± 153	527 ± 65	161 ± 25*
	3	3	2271 ± 352	545 ± 77	383 ± 93
	8	3	2379 ± 282	564 ± 66	160 ± 16*
	14	3	2486 ± 231	509 ± 60	138 ± 15
Cal^{-} Zn^{+} PLA^{-}	1	3	2122 ± 308	512 ± 24	218 ± 73
	3	3	2175 ± 261	536 ± 89	369 ± 102
	8	3	2402 ± 19	563 ± 82	168 ± 36
	14	3	2217 ± 71	583 ± 1*	133 ± 17
Cal^{-} Zn^{+} PLA^{+}	1	3	2183 ± 183	548 ± 4	155 ± 44
	3	3	2305 ± 62	558 ± 54	290 ± 36
	8	3	1983 ± 79	534 ± 28	169 ± 60
	14	3	2024 ± 256	524 ± 93	124 ± 11
Epogin	1	3	2626 ± 303	548 ± 81	233 ± 29
	3	3	2242 ± 161	484 ± 48	377 ± 46
	8	3	2155 ± 281	499 ± 76	116 ± 13
	14	3	2031 ± 173	526 ± 7	132 ± 19
Carrier alone	1	3	2120 ± 237	480 ± 36	118 ± 12*
	3	3	2128 ± 50	450 ± 31	147 ± 9*
	8	3	2257 ± 178	537 ± 75	124 ± 16
	14	3	2184 ± 330	543 ± 61	129 ± 10
Saline	1	3	1982 ± 266	482 ± 64	120 ± 12*
	3	3	2058 ± 299	490 ± 19	133 ± 12*
	8	3	2110 ± 39	543 ± 47	116 ± 12
	14	3	2228 ± 141	507 ± 23	123 ± 20

Values represent mean ± SD. *: $P<0.05$, versus Epogin.

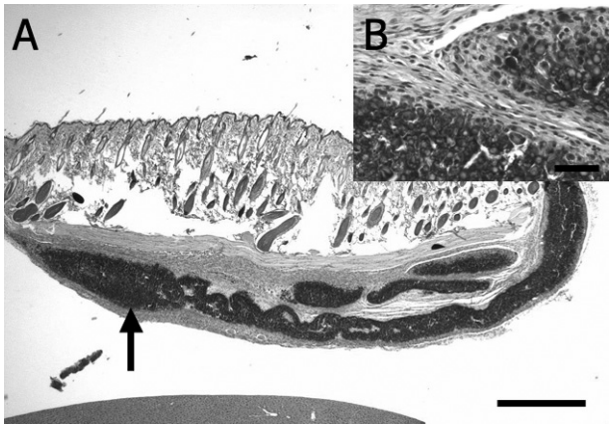

Fig. 6. Histopathology of the dorsal neck subcutaneous tissue on day 14 post-administration in Cal^{-} Zn^{+} PLA^{+}-administered mice. (A) Zn-HAp particles indicated by the arrow remained in the subcutaneous tissue on day 14. The bar represents 1 mm. (B) Tissue reactions were limited to the area in which foreign bodies were present, and were accompanied by minimal cellular infiltration and encapsulation by fibrous membranes. The bar represents 50 μm.

severe CKD is accompanied by anemia due to a lack of EPO [12, 13, 17, 36]. For CKD patients with renal anemia, rHuEPO has contributed to an improved quality of life and is known to protect against ischemic injury to the brain, spinal cord, retina, kidney, and myocardium [16]. However, the clinical application of rHuEPO demands patients undertake a troublesome treatment schedule requiring multiple administrations each week [9]. Therefore, the development of a novel DDS for EPO in the treatment of renal anemia is very necessary.

In DDS research, poly(lactic glycolic acid) (PLGA) has often been used as a carrier for the sustained release of therapeutic agents [2, 10, 28, 32, 33]. PLGA and block copolymer microspheres incorporating EPO formulated by a microencapsulation method have shown the sustained release of EPO for between two weeks and one month in an *in vitro* test [20, 21, 24]. There are, however, still problems with this carrier system in terms of deactivation, denaturation, inflammation, degradation of carriers, and insufficient content of therapeutic agents [3, 4, 22]. More recently, a new poly(ethylene glycol)-based copolymer was applied to the sustained release of EPO *in vivo* and it was demonstrated that the EPO concentration in plasma and hematocrit values were prolonged for two weeks [25]. However, synthetic polymers also raise some problems including protein denaturation. Naturally occurring polymer-based carriers, such as hyaluronic acid and gelatin, for the sustained release of EPO have been studied. Based on their network structure of polymer crosslinkings, hyaluronic acid hydrogels have shown the sustained release of EPO for 7 days *in vivo* [7], and gelatin-based sheets, which are applicable to the new

treatments for ischemic cardiomyopathy, sustained the release of EPO for 4 weeks [16].

In the present study, we demonstrated the efficiency of HAp in the sustained release of EPO. Zn-HAp microparticles formed using a spray-drying technique and Zn-HAp calcined at 400°C were used as carriers of EPO, and five Zn-HAp samples incorporating EPO were prepared; no formulation ($Cal^- Zn^- PLA^-$), PLA formulation ($Cal^- Zn^- PLA^+$), Zn formulation ($Cal^- Zn^+ PLA^-$), Zn/PLA formulation ($Cal^- Zn^+ PLA^+$), and calcined/Zn/PLA formulation ($Cal^+ Zn^+ PLA^+$) samples. We then examined which formulation was the most effective in sustaining EPO release *in vivo*. It is known that the protein absorbed into HAp is resistant to release *in vitro* because the protein absorbed into HAp is released with biodegradation of HAp. However, the detail mechanism is still unknown. Nagao *et al.* revealed human growth hormone absorbed into HAp is resistant to release in water, however it was released *in vivo* [22]. Similar phenomenon had occurred in the present study, so we could not measure the biological units of EPO/Zn-HAp. In preliminary studies, when 500 U of EPO/Zn-HAp was injected into dorsal neck subcutaneously, these mice showed the sustained efficacy of hemopoiesis. Thus, we used this dosage (500 U/body) in the present study. The dose of EPO administered to the mouse in two weeks was 8–40 fold of therapeutic human maintenance dose.

The efficiency of the sustained release was the lowest in the $Cal^- Zn^- PLA^-$-administered mice among the five samples. For the other samples, peak hematopoiesis was delayed and higher hematological values remained until day 14. Further, plasma EPO levels were higher for these samples than that for Epogin on day 3, particularly for the PLA formulation, which showed a reduction in the initial burst and plasma EPO levels remained high until day 8 in the $Cal^- Zn^+ PLA^-$ and $Cal^- Zn^+ PLA^+$ groups. In addition, their sustaining rates were higher than that of Epogin. This indicates that sustained EPO release could be achieved by using the Zn and/or PLA formulations on the Zn-HAp microparticles.

In transgenic mice overexpressing EPO, splenomegaly, an increase in spleen weight accompanied by an expansion of red pulp, was observed, suggesting the presence of extramedullary hematopoiesis [30]. In the present study, this same phenomenon was observed at a modest level in the mice administered Epogin, $Cal^- Zn^- PLA^-$, $Cal^- Zn^+ PLA^-$ and $Cal^- Zn^+ PLA^+$. In the present study, the relevance of the reduction of initial burst of EPO and the extramedullary hematopoiesis in spleen was suggested. The modest increase in spleen weight observed in $Cal^- Zn^+ PLA^+$ may be due to the lowest initial burst of EPO release compared to the other formulations. Thus, efficiency of sustained release of EPO with reduction of initial burst was suggested by inhibition of increase in spleen weight in $Cal^- Zn^+ PLA^+$.

Macroscopic observation showed that $Cal^- Zn^- PLA^-$, $Cal^- Zn^+ PLA^-$, and $Cal^- Zn^+ PLA^+$ still remained in the dorsal neck subcutaneous tissue on day 14, all samples never caused significant inflammatory reactions. Thus, tissue reactions were limited to the area in which foreign bodies were present, and were accompanied by minimal cellular infiltration and encapsulation by fibrous membranes. Although the biodegradability of HAp microparticles was examined after subcutaneous injection into rats [19], the examination for long-term degradation of Zn-HAp remaining in the dorsal neck subcutaneous tissue are necessary to examine the adverse long-term effects and the excretion mechanism.

In conclusion, the Zn-HAp microparticles were found to be useful carriers for the sustained release of EPO without serious adverse reactions, and the Zn/PLA formulation based the Zn-HAp microparticles was the most effective combination for the sustained release of EPO. In addition, the Zn-HAp microparticles showed good biocompatibilities as implants for as long as 14 days. Although young age healthy mice were employed in the present study, further study using adult age anemic model, such as ICR-derived glomerulonephritis (ICGN) mouse, drug-induced, antibody-induced, and nephrectomized animal models, will be necessary to examine the more accurate efficiency of hematopoiesis and the improvement of anemia by exogenous EPO.

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