

Amelioration of Anemia in the ICGN Mouse, a Renal Anemia Model, with a Subcutaneous Bolus Injection of Erythropoietin Adsorbed to Hydroxyapatite Matrix

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ABSTRACT. The recombinant human erythropoietin (rhEPO) is used for the treatment of patients with renal anemia. However, rhEPO should be administered subcutaneously or intravenously three times a week. The repetitive injections of rhEPO result in burdens to patients. To resolve this problem, we investigated the sustaining release methods using an rhEPO-hydroxyapatite (HAP) made by spray-drying technique as the drug delivery system. Two types of rhEPO-HAP formulations were prepared; zinc (Zn) formulation and Zn and poly-L-lactic acid (PLA) formulation. These formulations were examined in genetically anemic model, ICGN (ICR-derived glomerulonephritis) mice. According to *in vivo* release test of rhEPO from HAP in ICGN mice, elevated plasma concentration of rhEPO could be maintained for more than 7 days. These mice showed the amelioration of anemia for more than 3 weeks post-administration without causing any side effect. In conclusion, Zn or Zn/PLA formulation of HAP was considered to be one of the useful carriers of rhEPO for long-term improvement of anemia.

KEY WORDS: drug delivery system, erythropoietin, hydroxyapatite, ICGN mice, renal anemia.

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Chronic kidney disease (CKD), commonly also known as chronic renal disease, is a progressive and permanent loss of kidney function over a period of months or years. More than 275,000 patients are receiving dialysis treatment in Japan, and that 10 thousands of new patients with CKD increase per year [Japanese Society for Dialysis Therapy, http://www.jsdt.or.jp/index_e.html]. The anemia is a universal complication of CKD, because renal tubular dysfunction causes reduction of erythropoietin (EPO) responsible for red blood cell (RBC) production in kidney [14, 15, 17, 34], and approximately 80–90% of those are treated with recombinant human EPO (rhEPO) preparations. The rhEPO is used for the treatment of patients not only with renal anemia but also with many types of anemia caused by diseases interfering with RBC production, such as cancer, rheumatoid arthritis and so on [9]. However, rhEPO should be administered subcutaneously or intravenously three times a week to patients to maintain effective serum levels of EPO, because the sustained stimulation of the bone marrow by EPO is necessary [12]. For patients receiving rhEPO, the repetitive injections of rhEPO result in compliance problems of patients due to the burdens. To resolve this problem, the method of sustaining release of biologically active rhEPO over a period of 2 weeks or more should be necessary [22].

In the research filed for drug delivery system (DDS), biodegradable polymers have been used as a carrier for the sustained release. A single injection of rhEPO microencapsulated into poly(lactic-co-glycolic acid) microspheres to mice resulted in elevation of the number of RBCs, which would be equivalent to twelve injections of the rhEPO solution [7]. The rhEPO microspheres made by triblockcopolymers, of which polyethylenoxide blocks were sandwiched between poly(L-lactic-co-glycolic acid) blocks, provided a prolonged release of rhEPO up to 15 days *in vitro* [22]. A hydrogel prepared by *in situ* cross-linking of a thiol-containing poly(ethylene glycol)-based copolymer showed sustained release of rhEPO with higher hematocrit value for 2 weeks [27]. For naturally occurring-polymer-based carriers, such as hyaluronic acid and gelatin, the sustained release of rhEPO have been studied. Based on their network structure of polymer crosslinkings, hyaluronic acid hydrogels have shown the sustained release of rhEPO for 7 days *in vivo* [9, 23]. Gelatin-based sheets, which are applicable to the new treatments for ischemic cardiomyopathy, released rhEPO for 4 weeks [16]. Percutaneous administration of self-dissolving micropile made from dextrin provided a release of rhEPO for 10 days [10]. However, there are still problems with these carrier systems in terms of deactivation, denaturation, inflammation, degradation of carriers, aggregation of carriers, and insufficient content of therapeutic agents [4, 5, 7, 24].

Previously, we examined the efficiency of hematopoiesis using five hydroxyapatite (HAP) formulations for the sus-

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tained release of rhEPO *in vivo* [25]. As a result, in mice administered zinc (Zn) formulation and Zn and poly-L-lactic acid (PLA) formulation, plasma rhEPO was detected for a long time, peak hematopoiesis was prolonged, and higher hematological values were achieved. These results suggest that these formulations are useful matrix for the sustained release of rhEPO.

The ICGN (ICR-derived glomerulonephritis) mouse is an inbred strain showing the hereditary nephrotic syndrome due to a mutation of the *tensin2* gene [3]. With the deterioration of renal function, ICGN mice developed a normochromic and normocytic anemia, which is consistent with clinical reports on patients with renal anemia [32]. The expression of EPO mRNA in the kidneys was significantly reduced in ICGN mice [32]. Thus, ICGN mouse is an authentic model for human anemia with CKD [19, 31, 32]. In the present study, we examined whether these two formulations could ameliorate the severe anemia in ICGN mice more efficiently compared with aqueous rhEPO.

MATERIALS AND METHODS

Materials: Two types of zinc-containing HAp (Zn-HAp) formulation adsorbed rhEPO, Zn and Zn/PLA formulations, were prepared using the process described previously [25].

Animals: Specific pathogen-free ICGN were provided by National Institute of Biomedical Innovation (Osaka, Japan). Specific pathogen-free ICR female mice (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The anemic mice were produced by mating female (ICR \times ICGN) F_1 mice with male ICGN mice according to the previous reports [19, 31, 32]. Next, urine from each mouse was collected and proteinuria was measured using a urinary dipstick (Bayer Medical, Tokyo, Japan). The mice exhibiting proteinuria were used as anemic mice in this study. The mice exhibiting high proteinuria ($>1,000$ mg/dl) certainly showed anemia, whereas mice without proteinuria (<30 mg/dl) were normal heterozygotes, which were used for control in this study. They were housed in plastic cages under standard laboratory conditions (temperature: 20–26°C, relative humidity: 30–80%, light-dark cycle: 12/12 hours) in the animal facility of Japan Food Research Laboratories. They were given a CRF-1 diet (γ -rays irradiated, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. The present 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 animal experiment ethics committee of Japan Food Research Laboratories.

Hemanalysis, weight change of organs, and histopathological analysis post-administration: The two types of formulations suspended in saline or aqueous rhEPO (Epogin, Chugai Pharmaceutical, Tokyo, Japan) as a control were administered subcutaneously in the dorsal neck at the dose of 500 U/body to anemic mice, according to our previous report [25]. At least 50 U of rhEPO was able to absorb to 1 mg of HAp. In previous studies, when 500 U of rhEPO-

HAp injected subcutaneously, these mice showed the sustained efficacy of hemopoiesis without any side effects [25]. Thus, we used this dosage (500 U/body) to compare between both rhEPO-HAp and aqueous rhEPO in the present study. Carrier alone (Zn/PLA formulation without rhEPO) or saline was also administered subcutaneously in the same way. In male anemic mice (11–28 weeks old, $n=5$) and male control mice (11–15 weeks old, $n=5$), blood samples were collected from the postcava under anesthesia with ether and used for hematological analyses and enzyme-linked immunosorbent assay (ELISA) using a Human EPO ELISA Kit (Stemcell Technologies, Vancouver, BC, Canada) for plasma rhEPO concentration. Hematological analyses were performed with an automatic counter (KX-21NV; Sysmex, Kobe, Japan) according to the instruction manual. Reticulocytes were counted in the blood smears on glass slides stained with Brecher's New Methylene Blue Solution (Muto Pure Chemicals, Tokyo, Japan) and ratio of reticulocytes was calculated. The number of RBCs, hemoglobin concentration, hematocrit value, and the sustaining rate, which is the ratio of the value on day 21 to that on day 0, were evaluated as the sustained efficacy of hematopoiesis. Afterward they were sacrificed by excess ether inhalation and their enucleated liver, kidneys, and spleen were weighed. Liver and spleen as the hematopoietic organ and subcutaneous tissue as the administration site were fixed in 10% neutral-buffered formalin, and histopathological analyses were performed after hematoxylin and eosin staining.

Statistical analysis: Analysis of variance (ANOVA) was performed among three groups, Zn formulation, Zn/PLA formulation, and aqueous rhEPO using SPSS 11.0J for Windows (SPSS Japan Inc., Tokyo, Japan). When there was significant difference in ANOVA, the Tukey test was performed between two groups. These values were represented as means \pm SD and differences with $P<0.05$ were considered to be significant.

RESULTS

The mice exhibiting higher proteinuria showed severe anemia: It was examined whether the mice exhibiting high proteinuria were appropriate as anemic mice. Figure 1 shows the comparison of hematological parameters in mice between exhibiting higher proteinuria and without proteinuria. In mice exhibiting high proteinuria, the number of RBCs, hemoglobin concentration, and hematocrit value were lower by approximately 20% than that in mice without proteinuria ($P<0.05$, Fig. 1A, B, and C). Therefore, the mice exhibiting high proteinuria were useful for the anemic mouse model as described in the previous reports [19, 31, 32].

Hemanalyses in mice administered formulations: Zn and Zn/PLA formulations were compared with aqueous rhEPO as the control for long-term improvement for anemia. Figure 2 and Table 1 show the changes in hematological parameters post-administration in anemic mice. The number of RBCs, hemoglobin concentration, and hematocrit value in

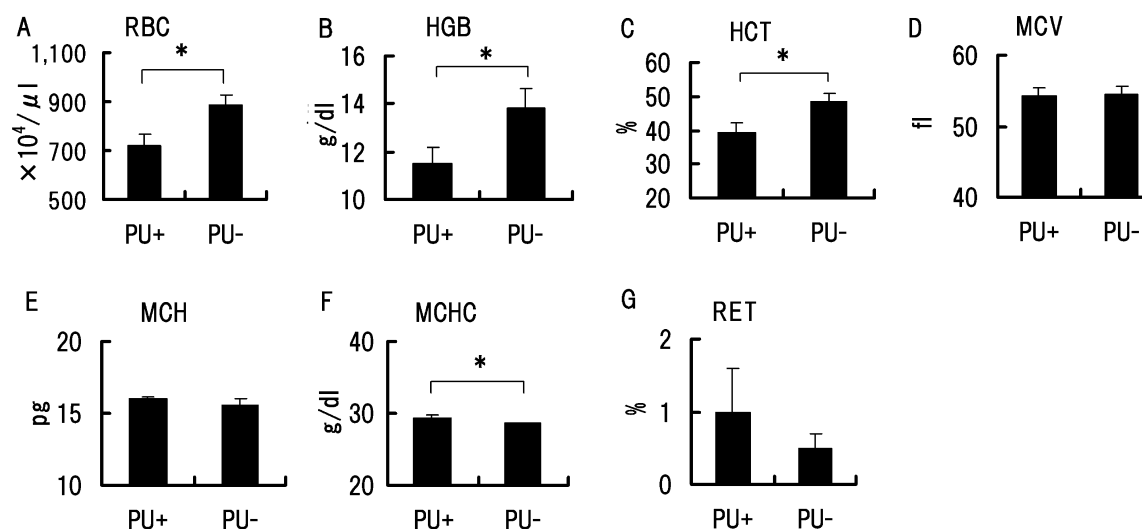


Fig. 1. Hematological parameters on mice exhibiting high proteinuria (PU+) and mice exhibiting low proteinuria (PU-). The number of red blood cells (RBC) (A), hemoglobin concentration (HGB) (B), hematocrit value (HCT) (C), mean corpuscular volume (MCV) (D), mean corpuscular hemoglobin (MCH) (E), mean corpuscular hemoglobin concentration (MCHC) (F), and ratio of reticulocytes (RET) (G) were determined. *, $P < 0.05$, PU+ versus PU-. The normochromic and normocytic anemia was observed in mice exhibiting high proteinuria.

mice administered aqueous rhEPO were broadly flat from day 0 to 14 and slightly decreased on day 21. In contrast, in mice administered both formulations, the significant hematopoietic improvements were observed on days 7, 14, and 21 post-administration, especially Zn formulation was more effective than Zn/PLA formulation ($P < 0.05$, Fig. 2A, B and C). The sustaining rate, the ratio of the value on day 21 to that on day 0, was calculated. The sustaining rates in the mice administered aqueous rhEPO was less than 100%, whereas those of mice administered both formulations were more than 110% (Fig. 3). These results are supported by the detection of plasma rhEPO in both formulations (Table 1). Although any plasma rhEPO was not detected in mice administered aqueous rhEPO, plasma rhEPO was detected in mice administered Zn formulation until day 14 and in mice administered Zn/PLA formulation until day 7, respectively. In mice administered both formulations, the time-dependent decrease in the ratio of reticulocytes was observed after transient increase of it on day 7, suggesting the improvement of anemia (Table 1). In contrast, in mice administered aqueous rhEPO, the increase in the ratio of reticulocytes was observed at day 21 after transient decrease of it, suggesting that the anemia was not completely cured. The significant differences in MCHC (hemoglobin concentration/hematocrit value $\times 100$) may be due to the elevation of the number of reticulocytes. Table 2 shows body weight change of mice post-administration. Mice administered both formulations showed favorable growth, whereas body weight of mice administered aqueous rhEPO decreased after day 14.

Examination of adverse effects on hematopoietic organ: To further characterize the response to rhEPO in formu-

administered mice, the weights of liver, kidneys, and spleen were measured to examine the adverse effects on hematopoietic organs. Figure 4 shows the differences in weights of the liver, kidneys, and spleen after administration. There was no significant difference between each group except for a slight difference in the spleen. Mice administered both formulations had slightly larger spleens than those of aqueous rhEPO-administered counterparts. This is most likely to be a result of extramedullary hematopoiesis and vascular congestion caused by the increase in RBC load.

Examination of histopathology: Dorsal subcutaneous tissues of the administration site were pathologically examined with respect to the tissue reaction around Hap particles. Both formulations were surrounded by fibrous membranes accompanied by fibroblasts and cellular infiltrations until day 21. Neither degeneration nor necrosis occurred in the injected sites (Fig. 5). Liver and spleen were pathologically examined with respect to the adverse effects on hematopoietic organ. Histopathological observation of the spleen in mice administered both formulations and aqueous rhEPO showed an expansion of red pulp with extramedullary hematopoiesis predominantly on day 7, and it was abolished by day 14, whereas the degree of expansion of red pulp in mice administered aqueous rhEPO was milder than that in mice administered both formulations (data not shown). In liver, no obvious abnormalities were found in all mice (data not shown).

DISCUSSION

EPO, a hematopoietic cytokine, is efficient drug for anemia caused by CKD, cancer, rheumatoid arthritis, HIV

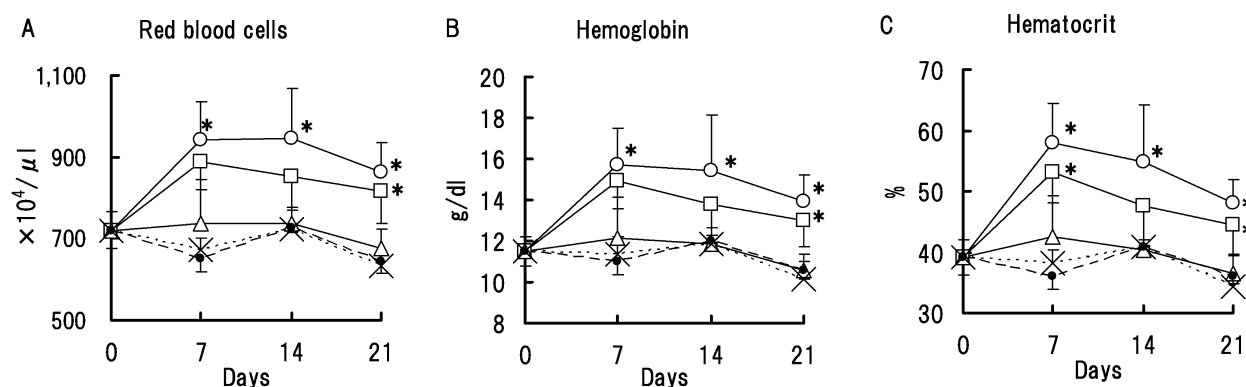


Fig. 2. Hematological parameters post-administration. The number of red blood cells (A), hemoglobin concentration (B), and hematocrit value (C) were determined. ○; Zn formulation, □; Zn/PLA formulation, △; aqueous rhEpo as a control, ●; carrier alone, and ×; saline. *, P < 0.05, versus aqueous rhEpo. In Zn and Zn/PLA formulations, the efficacy of hematopoiesis was observed until day 21.

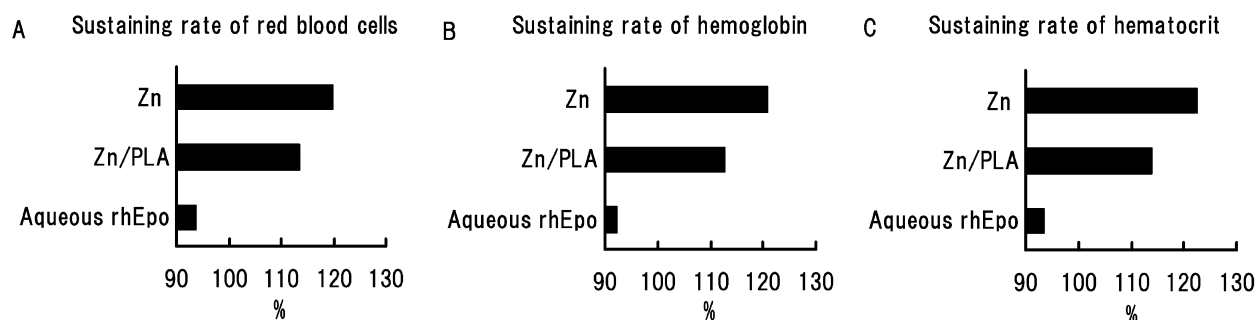


Fig. 3. Sustaining rate, the ratio of the value on day 21 to that on day 1, of the number of red blood cells (A), hemoglobin concentration (B), and hematocrit value (C). In Zn and Zn/PLA formulations, sustaining rates were higher than that of aqueous rhEpo as a control.

infection, ulcerative colitis and so on [9]. Since the site of EPO production is the peritubular cell of the kidneys, severe CKD is mostly accompanied by anemia due to a lack of EPO [14, 15, 17, 34]. The rhEPO is very effective for the CKD patients with renal anemia [1]; however, the clinical application of rhEPO demands patients to undertake troublesome treatment schedule requiring multiple administration in a week [12], which is a huge burden for both patients and medical staff. Thus, the development of a novel treatment method for anemia with DDS sustaining release of rhEPO for more than 2 weeks becomes necessary [22]. However, previous attempts to develop sustained release of rhEPO were not very successful. The present study revealed that a subcutaneous bolus injection of two formulations of rhEPO have the ability to improve anemia of ICGN mice, a congenital renal anemia model, for 3 weeks without serious adverse reactions.

In the present study, we have demonstrated new DDS via a HAp injection into skin tissue. Advantages of this skin-targeted approach include the fact that HAp administration is minimally invasive and have neither cellular nor humoral immune response. HAp particles have been examined as a carrier for sustained release of various therapeutic agents,

such as antibiotics [2, 13, 28, 29], anticancer drugs [11, 33], and proteins [6, 8, 18, 21]. It is known that the protein adsorbed to HAp is difficult to release *in vitro*, because the protein adsorbed to HAp is released with biodegradation of HAp [24]. Since similar phenomenon occurred in the present study, we could not measure the biological units of rhEPO-Hap *in vitro*. Previously, we examined the efficiency of hematopoiesis with five Zn-HAp formulations adsorbed rhEPO (no formulation, PLA formulation, Zn formulation, Zn/PLA formulation, and calcination/Zn/PLA formulation) *in vivo*. When 500 U of rhEPO-HAP was injected subcutaneously in the dorsal neck, Zn and Zn/PLA formulations only showed sustained efficiency in the hematopoiesis and plasma rhEPO levels [25]. Therefore, these 2 formulations were examined their efficiency for the improvement of anemia using ICGN mice, renal anemia model.

ICGN mice are inbred strain derived from ICR mice with hereditary nephrotic syndrome [20, 26, 32]. Previously we identified a mutation of the *tensin2* gene is responsible for the nephrotic syndrome of ICGN mice [3]. The anemia of ICGN mice is normochromic and normocytic anemia, which is, thus, consistent with clinical reports on patients

Table 1. Hematological data post-administration

Sample	Days	n	Week age	WBC ($\times 10^2/\text{ml}$)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plasma rhEPO (mU/ml)	RET (%)
Zn formulation	7	5	15 \pm 8	96 \pm 49	61.5 \pm 3.0	16.7 \pm 0.9	27.2 \pm 0.6*	1.4 \pm 0.9	3.6 \pm 1.9
	14	5		99 \pm 39	57.7 \pm 2.8	16.2 \pm 0.9	28.1 \pm 0.4*	0.3 \pm 0.3	0.8 \pm 0.6
	21	5		98 \pm 13	55.6 \pm 1.0	16.1 \pm 0.4	28.9 \pm 0.5	ND	0.3 \pm 0.4*
Zn/PLA formulation	7	5	13 \pm 4	85 \pm 49	59.9 \pm 1.8	16.8 \pm 0.5	28.1 \pm 0.6	0.5 \pm 0.7	3.8 \pm 3.4
	14	5		89 \pm 21	56.0 \pm 1.5	16.2 \pm 0.5	29.0 \pm 0.6	ND	0.9 \pm 0.5
	21	5		72 \pm 36	54.5 \pm 2.0	15.9 \pm 0.4	29.2 \pm 0.7	ND	0.6 \pm 0.3*
Aqueous rhEpo	7	5	14 \pm 7	65 \pm 23	57.5 \pm 1.7	16.4 \pm 0.6	28.6 \pm 0.8	ND	2.9 \pm 2.5
	14	5		60 \pm 12	55.0 \pm 1.3	16.1 \pm 0.6	29.3 \pm 0.9	ND	1.1 \pm 0.2
	21	5		72 \pm 13	54.3 \pm 1.5	15.7 \pm 0.7	29.0 \pm 1.4	ND	2.7 \pm 0.9
Carrier alone	7	3	13 \pm 2	73 \pm 9	55.3 \pm 0.8	16.9 \pm 0.4	30.4 \pm 0.3	ND	0.4 \pm 0.1
	14	3		65 \pm 29	56.2 \pm 0.6	16.5 \pm 0.1	29.3 \pm 0.1	ND	0.6 \pm 0.1
	21	3		110 \pm 44	56.0 \pm 0.8	16.4 \pm 0.2	29.3 \pm 0.1	ND	0.7 \pm 0.2
Saline	7	3	13 \pm 1	62 \pm 15	56.9 \pm 3.0	16.9 \pm 0.6	29.8 \pm 0.6	ND	0.3 \pm 0.1
	14	3		52 \pm 24	56.4 \pm 1.2	16.4 \pm 0.2	29.0 \pm 0.3	ND	0.6 \pm 0.1
	21	3		91 \pm 47	54.1 \pm 1.6	16.1 \pm 0.8	29.7 \pm 0.7	ND	0.8 \pm 0.1

Values represent mean \pm SD. *: $P < 0.05$, versus aqueous rhEpo. WBC: White blood cells, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, RET: Rate of Reticulocytes. ND: Not detected.

Table 2. Body weight change post-administration

Sample	Day 0 (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)
Zn formulation	31.2 \pm 5.1 (n=15)	32.3 \pm 4.7 (n=15)	32.4 \pm 5.8 (n=10)	34.3 \pm 3.2 (n=5)
Zn/PLA formulation	30.8 \pm 3.4 (n=15)	32.0 \pm 3.6 (n=15)	33.3 \pm 2.9 (n=10)	34.4 \pm 1.5 (n=5)
Aqueous rhEpo	30.7 \pm 5.0 (n=15)	31.3 \pm 5.9 (n=15)	31.0 \pm 4.2 (n=10)	30.2 \pm 3.2 (n=5)
Carrier alone	29.4 \pm 3.1 (n=9)	29.8 \pm 2.9 (n=9)	28.9 \pm 3.2 (n=6)	30.3 \pm 3.8 (n=3)
Saline	29.5 \pm 2.9 (n=9)	30.0 \pm 2.7 (n=9)	29.8 \pm 3.0 (n=6)	30.1 \pm 1.6 (n=3)

Values represent mean \pm SD.

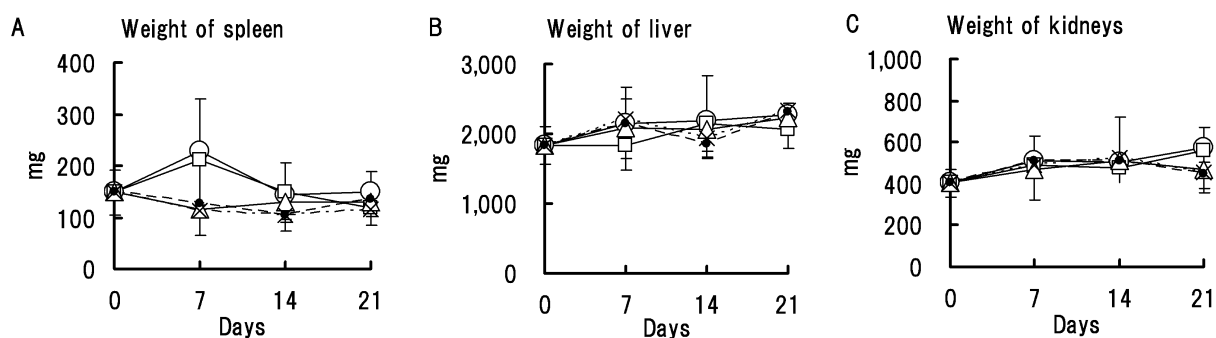


Fig. 4. Weight of organs post-administration. Weights of spleen (A), liver (B), and kidneys (C) were determined. \circ ; Zn formulation, \square ; Zn/PLA formulation, and \triangle ; aqueous rhEpo as a control, \bullet ; carrier alone, and \times ; saline. In Zn and Zn/PLA formulations, an increase of spleen weight was observed at day 7.

with renal anemia [32]. The anemia of ICGN mice is caused by decreased production of EPO in kidney. There are some reports for the treatment of anemia in ICGN mice with rhEPO, in which anemia in ICGN mice was improved by subcutaneous administration of rhEPO [19, 31]. Therefore, ICGN mice are appropriate renal anemic model compared with artificial models, such as drug-induced, antibody-

induced, and nephrectomized animal models [32].

Our previous report shows aqueous rhEPO-administered ICR mice, the peaks of all hematopoietic parameters were observed on day 3 post administration, whereas those of the rhEPO-HAP formulation-treated ICR mice were sustained up to day 8, indicating that rhEPO-HAP formulation sustained the effect of rhEPO on hematopoiesis. This was sup-

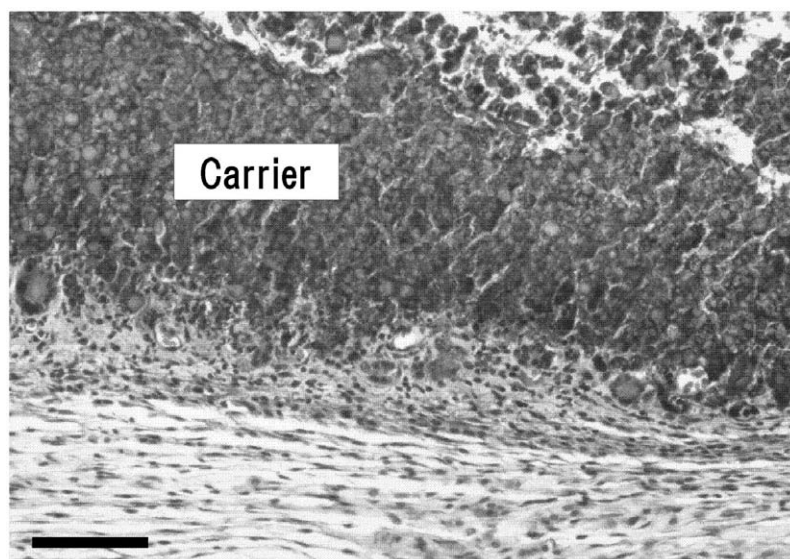


Fig. 5. Histopathology of dorsal subcutaneous tissue on day 21 post-administration in Zn formulation-administered mice. Zn-HAp particles (carrier) remained in the subcutaneous tissue on day 21. Tissue reactions were limited to the area in which foreign bodies were present, and were accompanied by minimal cellular infiltration and encapsulation by fibrous membranes. The bar represents 100 μ m.

ported by the data for plasma rhEPO levels [25]. In the present study, the hematological parameters after aqueous rhEPO administration in anemic ICGN mice were not different from that of day 0 or saline administrations. These data indicate that anemic ICGN mice might be resistant to rhEPO treatment similar to human CKD patients. As a matter of fact, aqueous rhEPO should be administered to patients three times a week to maintain suitable levels of serum rhEPO. The hematological parameters in ICGN mice administered both formulations increased and the peak of hematopoiesis was observed on day 7 and slightly decreased after day 14; however, they were always higher than that of aqueous rhEPO until day 21. These data suggest that both formulations are useful for sustaining the release of rhEPO *in vivo*.

Although macroscopic observation showed that both formulations still remained in the subcutaneous tissue on day 21, these formulations did not cause any significant inflammatory reactions. The biodegradability of HAp microparticles injected subcutaneously was observed in rats [21]. Thus, more detailed examinations for degradation of both formulations remaining in the subcutaneous tissue are necessary as well as the adverse long-term effects and the excretion mechanism to be studied. In transgenic mice overexpressing EPO, splenomegaly, an increase in spleen weight accompanied by an expansion of red pulp, was observed, suggesting the extramedullary hematopoiesis [30]. In the present study, the same phenomenon was observed in mice administered both formulations. In mice administered aqueous rhEPO, the modest expansion of red pulp with extramedullary hematopoiesis was observed; however, no

increase in spleen weight was observed. The sustained release of rhEPO from both formulations was also suggested by this result. Recently, novel erythropoiesis stimulating protein (NESP) made by conjugating sugar chains to rhEPO was developed. The half-life of NESP in blood is about three times longer than rhEPO. The retention time of efficacy for the EPO/HAp presented in this paper, however, seems to be the same as or longer than that of the NESP.

In conclusion, HAp was assessed as a novel drug carrier to achieve sustained release of rhEPO. According to *in vivo* release test of rhEPO from HAp in ICGN mice, elevated plasma concentrations of rhEPO were maintained for 7 days. There was no adverse effect during and after the administration. Further optimization study is necessary to achieve longer sustained release of rhEPO to establish curative DDS for anemia.

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