2. Materials and methods

2.1. Materials

Erythropoietin and granulocyte-colony stimulating factor (G-CSF) were kind gifts from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Betamethasone sodium phosphate (BSP) was purchased from Sigma Chemical Co. (St. Louis, MO). Male Sprague-Dawley rats were obtained from SLC Experimental Animals (Shizuoka, Japan) at 7 weeks of age. The body weights of rats at the time of experiments ranged from 186 to 230 g. The animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Jikei University School of Medicine.

2.2. Incorporation and absorption of betamethasone phosphate into or on nano-$CaCO_3$

Bethamethasone phosphate (BP) was incorporated into calcium carbonate nanoparticles (nano-$CaCO_3$) by the following method: 650 μl of 5M $CaCl_2$ and 375 μl of 5% BSP were gently mixed for 10 min, and then 2.5 ml of 1M Na_2CO_3 was added and stirred gently or vigorously for 10 min. After the addition of 5 ml of distilled water and discarding the large particles of nano-$CaCO_3$ which were precipitated without centrifugation, the suspension was centrifuged (2000 rpm, 5 min), and the supernatant and precipitate were separated. The amount of BP in the supernatant and precipitate was measured respectively with HPLC, after dissolving the $CaCO_3$ with 0.5M EDTA (pH 7.5). The fabricated nano-$CaCO_3$ containing BP were used for *in vitro* and *in vivo* experiments. We tested with different ratio in mole of $CaCl_2$/ Na_2CO_3 at 1/1.6, 1/1.3, 1, 1.3, 1.6 and observed that the incorporation rate was highest at the ratio of 1.3, and used the ratio in this study.

To study the adsorption of BP, we mixed 650 μl of 5M $CaCl_2$ and 2.5 ml of 1M Na_2CO_3 and stirred gently or vigorously, according to desired particle size, and nano-$CaCO_3$ were formed. After the particles were washed with 5 ml of distilled water, 375 μl of 5% BSP was added and stirred to adsorb BP. The resulting suspension was centrifuged (2000 rpm, 5 min), and the amount of BP in the precipitate was determined by the method described above.

2.3. Incorporation and adsorption of erythropoietin in or on nano-$CaCO_3$

Erythropoietin was incorporated into nano-$CaCO_3$ by mixing 650 μl of 5M $CaCl_2$ and 125 μl of 1 mg/ml erythropoietin, and then by adding 2.5 ml of 1M Na_2CO_3 stirring gently for 10 min at room temperature. After the addition of 5 ml of distilled water, the suspension was divided into 2 parts and centrifuged (2000 rpm, 5 min). One part was used for the measurement of erythropoietin, and the other part was washed by 0.9 ml of 1M Na_2CO_3 twice to eliminate the adsorbed erythropoietin on the nano-$CaCO_3$. The both samples were dissolved by adding 1N HCl, and the amount of incorporated erythropoietin in the nano-$CaCO_3$ was measured with an ELISA kit (Toyobo Co., Ltd., Osaka, Japan).

In the adsorption experiment with erythropoietin, 650 μl of 5M $CaCl_2$ and 2.5 ml of 1M Na_2CO_3 were mixed and stirred initially, and nano-$CaCO_3$ were formed. Next, the particles were washed by 5 ml of distilled water and 125 μl of 1 mg/ml erythropoietin was added and stirred to adsorb the erythropoietin. The resulting suspension was divided into 2 parts and centrifuged (2000 rpm, 5 min). The obtained precipitate of 1 part was used for the measurement of erythropoietin, and that of the other part was washed twice by 0.9 ml of 1M Na_2CO_3. The amount of erythropoietin of each part was determined by the method described above.

2.4. Particle size

Particle size of the prepared nano-$CaCO_3$ was measured with a laser light scattering method by using a fiber-optics particle analyzer (FPAR-1000, Otsuka

Electronics Co., Ltd, Osaka, Japan). The measurement was performed in triplicate, and the median size and range of distribution were obtained.

2.5 Release studies

2.5.1. Release of G-CSF from nano-$CaCO_3$ preparation in vitro

To incorporate G-CSF in nano-$CaCO_3$, we mixed 650 μl of 5M $CaCl_2$ and 250 μl of 500 μg/ml G-CSF, and added 2.5 ml of 1M Na_2CO_3 stirring gently for 10 min. After the addition of 5 ml of distilled water, the suspension was centrifuged (2000 rpm, 5 min) and the supernatant was discarded. The obtained precipitate was resuspended in 12.5 ml of 0.1M Tris-HCl buffer (pH 7.2) containing 1% bovine serum albumin (final concentration of G-CSF was approximately 1μg/ml) and shaken gently for 7 days at room temperature. For the measurement of G-CSF released from the preparations, 0.1 ml of the suspension was withdrawn and replaced with an equal volume of the same buffer. The samples were diluted and centrifuged (2000 rpm, 5 min) and amount of G-CSF in the obtained supernatant was measured with an ELISA kit (Immuno-Biological Laboratories Co., Ltd, Gunma, Japan). These operations were carried out once a day for 7 days. After the final sampling on day 7, the total suspension was centrifuged and the precipitated nano-$CaCO_3$ were dissolved by adding 1N HCl and the residual amount of G-CSF was measured. A stability test on free G-CSF was also performed. Fifty μl of 100 μg/ml G-CSF and 4.95 ml of the same buffer were mixed (final G-CSF concentration was 1.0 μg/ml) and shaken simultaneously at room temperature. The amount of G-CSF in the solution (20μl was taken) was measured with the ELISA kit once a day for 7 days.

2.5.2. Release of BP from nano-$CaCO_3$ preparation in vivo

We administered 0.5 ml of the BP-incorporated nano-$CaCO_3$ suspension subcutaneously in rats at a dose of 1 mg/head. As a control, 0.5 ml of aqueous solution of BSP was administered subcutaneously in rats at a dose of 1 mg/head as BP. Blood was sampled via the inferior ophthalmic vein from rats anesthetized with ether before and 0.5, 1, 3, 5, 7, 16, 24 and 48 hours after the administration. BP concentration in plasma was determined by a time-resolved fluoroimmunoassay.

2.6. Statistical analysis

The data derived from each experimental series are represented as the mean ±

SEM. The data were analyzed by using an ANOVA. Post hoc determination were assessed for statistical significance by using Student's *t* test [9].

3. Results

3.1. Loading (incorporation and absorption) of BP in or on nano $CaCO_3$ and particle size.

Particle size and loading efficiency of nano-$CaCO_3$ were examined (Table 1). The diameter of the particles was dependent on mixing speed; gentle stirring (ca. 650 rpm) produced large particles, while vigorous stirring (ca. 1300 rpm) produced small particles. The range of distribution in particle size was narrower in the experiment with vigorous mixing than that with gentle one. The loading efficiency in particles of the both sizes was high and similar in the incorporation experiment. The BP contents in the supernatant by gentle and vigorous stirring was 8.9% ± 1.7% and 9.4% ± 1.3% of the total BP used, respectively. The amount of loading of BP in the adsorption experiment was much lower than that in the incorporation experiment.

3.2. Incorporation and adsorption of erythropoietin in or on nano-$CaCO_3$

The diameter of the particles incorporating or adsorbing erythropoietin was 105.5 ± 11.2 nm and 128.0 ± 13.1 nm, respectively. The influence of washing on the release of erythropoietin was examined (Figure 1). More adsorbed erythropoietin, compared to incorporated erythropoietin, was released after the particles were washed.

3.3. Sustained release of G-CSF from nano-$CaCO_3$ preparation in vitro and stability of G-CSF in the particles and solution.

The amount of G-CSF released over time from the nano-$CaCO_3$ was cumulatively plotted in Figure 2. G-CSF was gradually released from the nano-$CaCO_3$ through 7 days and still remained in the nanoparticles on day 7. The amount (concentration) of G-CSF in the solution on day 7 was even smaller than that in the residue of the nano-$CaCO_3$, which indicated that G-CSF was not stable in the solution but was very stable in the nano-$CaCO_3$.

3.4. Sustained release of BP from nano-$CaCO_3$ preparation in vivo

Plasma concentration of BP after the subcutaneous injection of BP-incorporated nano-$CaCO_3$ and free BP solution in rats is shown in Figure 3. The nano-$CaCO_3$ used in the experiment were fabricated by gentle mixing. After the injection of BP solution, the plasma concentration of betamethasone was increased and then decreased steeply. While, after the injection of BP-incorporating nano-$CaCO_3$, the degree of initial increase in concentration was ~~low~~ not high and a subsequent sustained release was observed in compared with the betamethasone solution. A significant sustained release was shown 48 hours after the injection. However, an initial burst was observed even with the nano-preparation. It indicates that the release of BP from the nano-particles in subcutaneous tissue was rapid as compared with that in water (Table1) even in the incorporated form.

4. Discussion

In the present study, we devised a simple method of incorporating drugs into nano-$CaCO_3$ that can be utilized for a delivery system. The incorporating efficiency of drugs was sufficiently high for the practical use in the clinic. It was confirmed in *in vitro* and *in vivo* experiments that the nano-$CaCO_3$ preparations showed a sustained release of incorporated drugs. Although the experimental reports of drug-adsorbing $CaCO_3$ particles have been published [6,7,8], the methods of incorporating drugs into $CaCO_3$

have not. The incorporated drugs are apparently more effective in DDS than the adsorbed drugs, because $CaCO_3$ particles are dissolved very slowly in the body.

In this study, we incorporated drugs with ionic, hydrophilic residues and low molecular weight, such as BP, and bioactive proteins, such as erythropoietin and G-CSF. Our preliminary experiments have shown this method of incorporationg drugs to be practicable for low molecular weight compounds with a carboxyl residue, such as retinoic acid, those with a phosphate residue, such as hydrocortisone sodium phosphate, and those with a high molecular weight, such as immunoglobulin G and deoxyribonucleic acid (data not shown). Therefore, this method of incorporation is applicable to various drugs despite their molecular weight.

The diameters of nano-$CaCO_3$ incorporating BP were varied from 40 to 200 nm by changing the mixing speed (Table 1). Nanoparticles with a size of 50 nm or 200 nm have been reported to accumulate in angiogenic blood vessels [1,10], tumor [11] and inflamed tissues [3,12]. Thus, nano-$CaCO_3$ with incorporated anti-cancer and anti-inflammatory drugs may demonstrate targeting effects to the specific regions. We have obtained a preliminary result that nano-$CaCO_3$ accumulated in an inflamed region (data not shown). In addition, nanoparticles with a proper size and chemically appropriate surface properties were reported to penetrate the skin [13] and the blood-brain barrier [14].

Furthermore, the particles with the smallest size in this study (about 40 nm) should be able to avoid phagocytosis by the reticuloendothelial system (RES) or cells [1,15]. Avoiding clearance by the RES is very important in DDS using nanoparticles. Therefore, several attempts such as a modification of particle surface [16] and a use of surface binding of polyethylenglycol (PEG) [2] were made.

The chemical stability of G-CSF was markedly increased with this incorporating method (Fig. 2). As well known, several bioactive proteins are unstable in the solution. We think that nano-$CaCO_3$ particles are also useful for the treatment with unstable bioactive proteins.

5. Conclusion

The present study describes a simple technology for incorporating drugs with low molecular weight and bioactive proteins into $CaCO_3$ nanoparticles, which can be used in various kinds of DDS.

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Figure Legends

Fig. 1. Influence of washing on the release of drug from the calcium carbonate nanoparticles (nano-$CaCO_3$) either incorporating or adsorbing erythropoietin. The abscissa represents the percentage of erythropoietin content compared with that measured before washing. Each column and horizontal bar represents the mean ±SEM of 3 experiments.

Fig. 2. *In vitro* release of granulocyte colony-stimulating factor (G-CSF) from calcium carbonate nanoparticles (nano-CaCO3) (closed circle) and inactivation of free G-CSF in buffer (open circle). Each symbol represents the mean of 3 experiments.

Fig. 3. Plasma concentration of betamethasone after the subcutaneous injection of calcium carbonate nanoparticles (nano-$CaCO_3$) incorporating betamethasone phosphate (BP) (closed circle) and inactivation of free G-CSF in buffer (open circle).

Each symbol represents the mean ± SEM of 3 experiments.

※ : significant difference ($p < 0.05$) between the 2 groups; † : significant difference ($p < 0.05$) in relation to 0 hour § : no significant difference ($p > 0.05$) in relation to 0 hour.

Table 1

Particle size of calcium carbonate nanoparticles (Nano-$CaCO_3$) loading betamethasone phosphate (BP) and their loading efficiency

Mixing speed	Particle size (nm)		Loading efficiency [a] (%)	
	Incorporation	Adsorption	Incorporation	Adsorption
Gentle ca. 650 rpm	140.7 ±21.4 (95.8 - 165.1)[b]	170.5 ±24.0 (139.5 - 209.5)	91.1 ±1.7	9.1 ±1.3
Vigorous ca. 1300 rpm	44.8 ±1.1 (35.3 - 56.8)	101.5 ±12.6 (80.9 - 131.7)	90.6 ±1.3	25.7 ±2.8

[a] Loading efficiency is the percentage of incorporated or adsorbed BP compared to the total BP used. Each value represents mean ±SEM of 3 experiments.

[b] Range of distribution in the particle size

Fig. 1. Mizushima

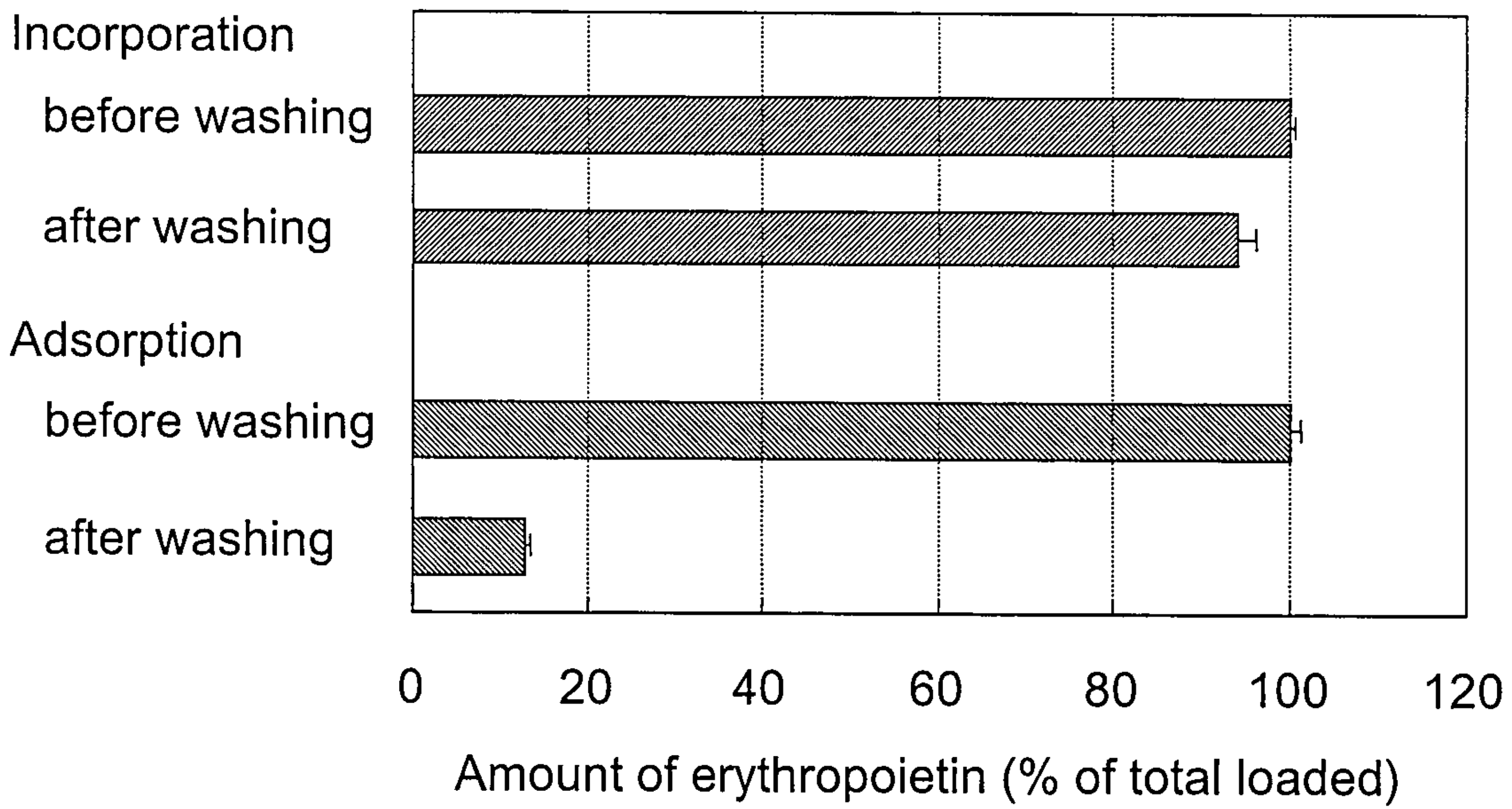


Fig. 2. Mizushima

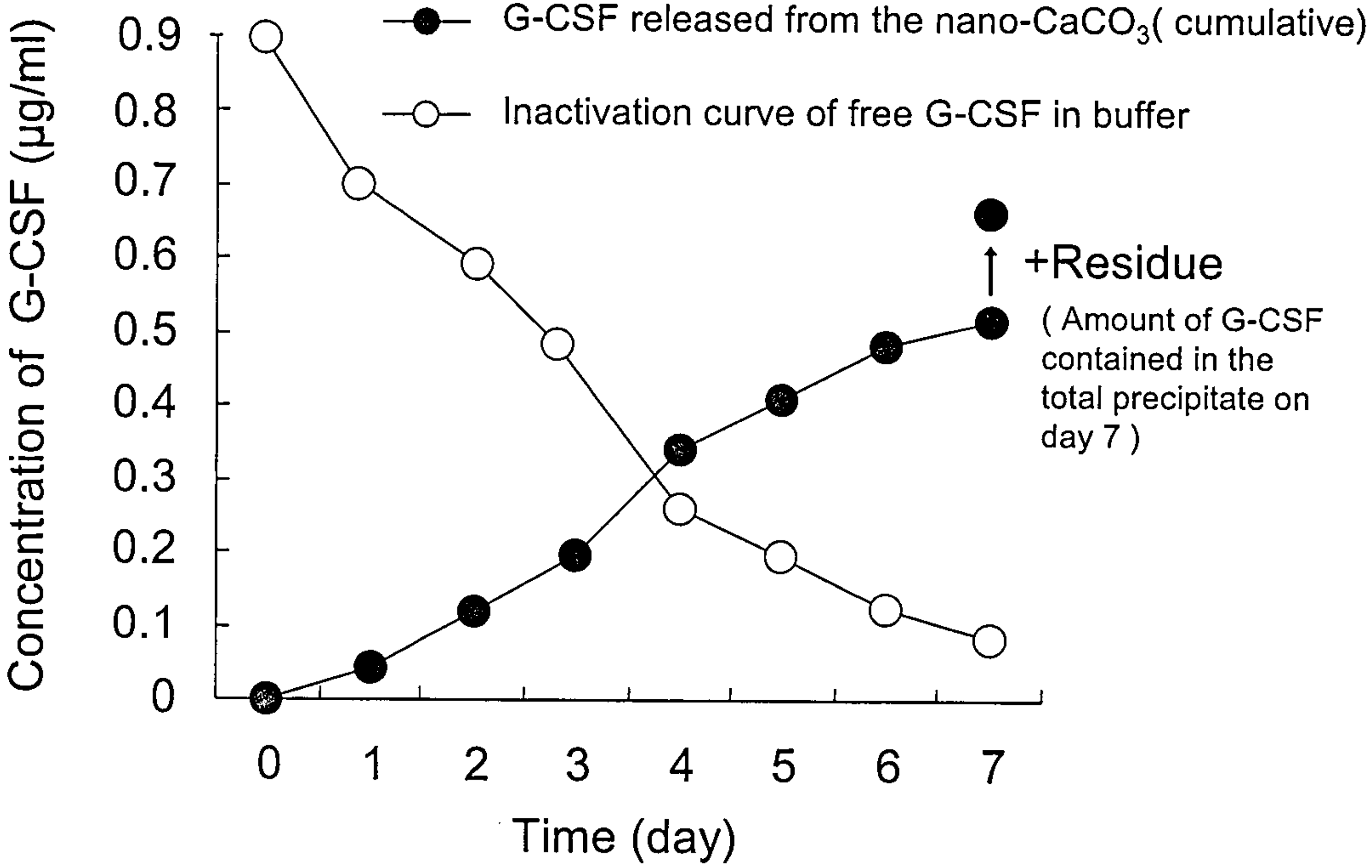
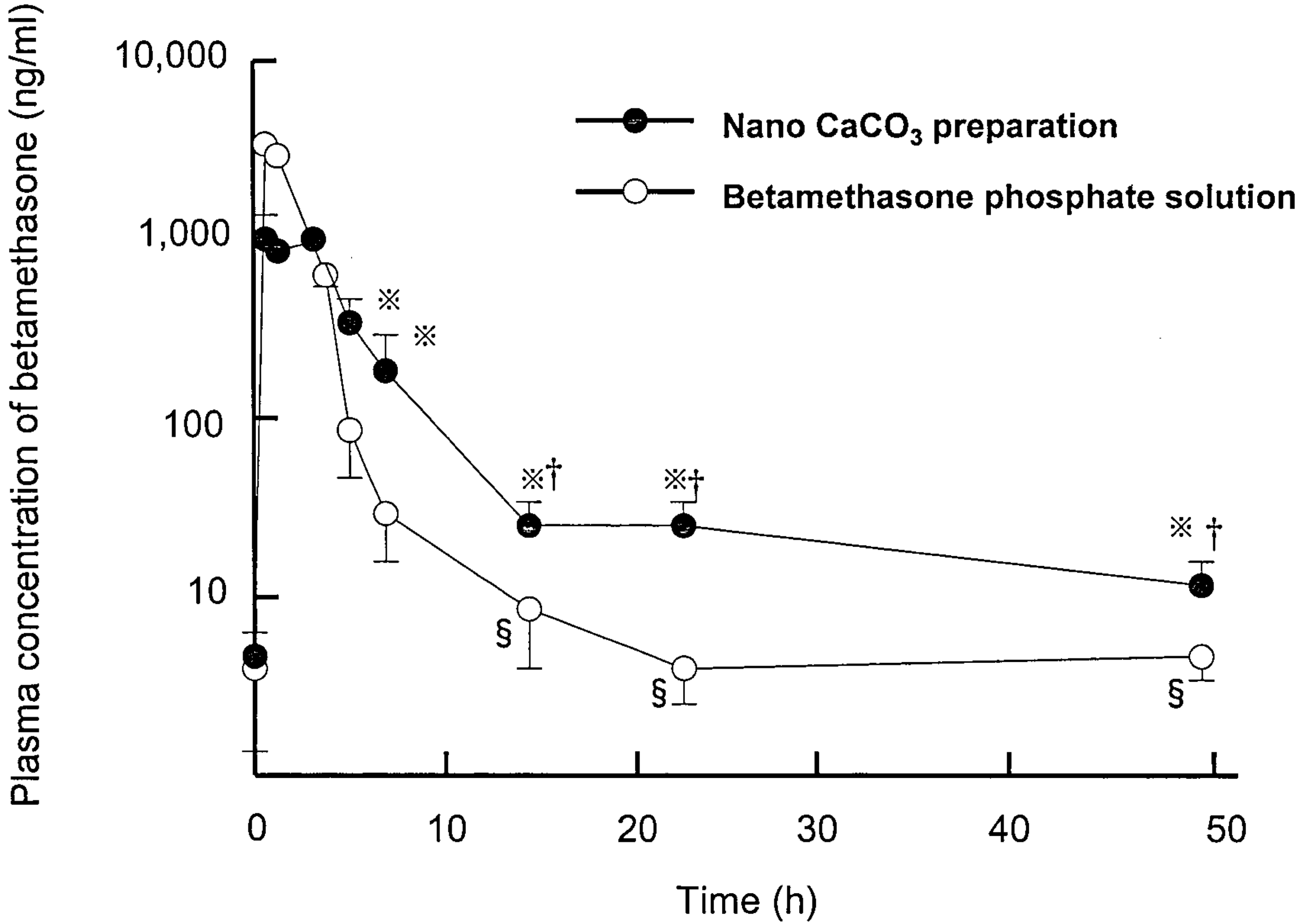


Fig.3. Mizushima



Injectable porous hydroxyapatite microparticles for the delivery of protein and lipophilic drugs

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