J Mater Sci: Mater Med (2007) 18:513–522
DOI 10.1007/s10856-007-2012-2

Subacute systemic toxicity assessment of β-tricalcium phosphate/carboxymethyl-chitin composite implanted in rat femur

Kazuaki Muramatsu · Kousuke Oba · Daisuke Mukai · Kazunari Hasegawa · Shingo Masuda · Yusuke Yoshihara

Received: 19 April 2005 / Accepted: 14 November 2005

Abstract The efficacy of a composite of β-tricalcium phosphate particles and carboxymethyl-chitin (β-TCP/CM-chitin) for bone repair has already been established in animal experiments. In the present study, subacute systemic toxicity was evaluated to further assess the biological safety of the implanted composite. β-TCP/CM-chitin (approximately 4 mg/kg and 7 mg/kg in male and female rats, respectively) was implanted for 28 days into penetrating defects (2 mm diameter) made artificially in the shaft of the right femur of rats. Sham operation groups with the defect only were prepared as controls. Haematology, blood chemistry, urinalysis, and the histopathology of 44 organs and tissues were investigated. Body weight measurements and clinical observations were performed daily throughout the study. No subacute systemic toxicity possibly caused by the implantation of β-TCP/CM-chitin was detected. These findings indicate that β-TCP/CM-chitin composite is a highly biocompatible bone substitute, at least with an implantation dosage of $<$4–7 mg/kg.

K. Muramatsu (✉)
Department of Biotechnology, School of Science and Engineering, Tokyo Denki University, Ishizaka, Hatoyama-cho, Hiki-gun, Saitama 350-0394, Japan
e-mail: muramatsu@b.dendai.ac.jp

K. Oba · D. Mukai · K. Hasegawa
Experimental Toxicology Department, Biosafety Research Center, Foods, Drugs and Pesticides, 582-2 Shioshinden, Fukude-cho, Iwata-gun, Shizuoka 437-1213, Japan

S. Masuda · Y. Yoshihara
Research and Development Corporate Division, Japan Medical Materials Corporation, 3-3-31 Miyahara, Yodogawa-ku, Osaka 532-0003, Japan

Introduction

Recently, tricalcium phosphates (TCPs) have been used clinically as bioabsorbable bone substitutes and as a scaffold for bone tissue engineering. The clinical application of β-TCP in the dental and orthopedic fields goes back to the 1970s, and the bone conductivity and biocompatibility of the material have been confirmed experientially [1–4]. However, there are some issues concerning the clinical use of β-TCP. For example, although its granules can fill variously shaped bone defects, the granules tend to fall out of the defects during surgery. A novel bioabsorbable bone substitute (β-TCP/CM-chitin), composed of β-TCP and carboxymethyl-chitin, which has a suitable TCP:CM-chitin ratio and plasticity, was developed to solve this problem [5]. The biodegradability of the CM-chitin in the composite was controllable by vacuum-heating [6], and this characteristic also supported the osteoconductivity of β-TCP [5, 7].

β-TCP/CM-chitin contains a novel component, CM-chitin, and biological safety evaluation of this material is mandatory. Previous unpublished data confirmed that this composite was completely biodegraded within a month in the femur of rabbits and in the tooth extraction sockets of dogs and Macaca fascicularis since CM-chitin was more susceptible to endogenous lysozyme than the other chitin derivatives [8, 9]. In our previous study, in vitro assays showed no genotoxicity or carcinogenicity in extracts of β-TCP/CM-chitin [7]. To investigate the biological safety of β-TCP/CM-chitin implanted into bone, further systemic toxicological evaluation was required.

In the present study, in order to study the biocompatibility of the β-TCP/CM-chitin composite, we evaluated the subacute systemic toxicity of the composite implanted in the femur for 28 days, using haematological, biochemical, and

histopathological observations in accordance with the Good Laboratory Practice (GLP) standard.

Materials and methods

Preparation of β-TCP/CM-chitin composite

CM-chitin powder (degree of substitution (DS) for O-carboxymethylation, degree of deacetylation (DD), weight-average molecular weight (Mw) approximately 80%, 30%, and 5×10^5, respectively) was purchased from Koyo Chemical Co. Ltd. (Osaka, Japan), and β-TCP powder (1–2 μm diameter) was purchased from the Taihei Chemical Industrial Co. Ltd. (Osaka, Japan).

The β-TCP/CM chitin composite was prepared using a previously reported method [7]. Briefly, CM-chitin was dissolved in Milli-Q water (purified to 18.2 MΩ cm) and precipitated in ethanol. The precipitate was then processed to enhance insolubility in water by heat-treatment in a vacuum (approximately 1.0–1.5 kPa) at 140°C for 12 h. The β-TCP powder was compressed into pellets, sintered at 1,100°C for 2 h, and ground into particles of approximately 50–150 μm in diameter. The β-TCP particles were mixed with vacuum heated CM-chitin and Milli-Q water (β-TCP: CM-chitin: water = 5:1:32 (wt%)). The suspension was then freeze-dried and sterilized by Co-60 γ-ray irradiation (25 kGy).

Animal experiments

SPF/BrlHan:WIST@Jcl(GALAS) rats (age 10 weeks) used in the present study were purchased from CLEA Japan, Inc. (Tokyo, Japan), and kept under a standard light-dark schedule and relative temperature and humidity for 7 days to adapt to their environment. Male (332–362 mg) and female (197–218 mg) rats were anaesthetized by intraperitoneal injection of pentobarbital sodium, and the right femur was exposed by incision. A columnar specimen was implanted into a penetrating defect (2 mm in diameter) made artificially in the center of the femoral shaft, as shown in Fig. 1. The amount of β-TCP/CM-chitin implanted into males and females was approximately 4 mg/kg and 7 mg/kg, respectively. After implantation of specimens using a 14-gauge syringe and stylet, connective tissues and skin were sutured. A sham operation without implantation into the bone defect was performed as a comparative control. Stock diet and tap water were available *ad libitum* until the evening preceding the day of sacrifice. Twenty-eight days after surgery, the haematological and histopathological characteristics of 44 organs and tissues were investigated for each rat. Urine was collected from each rat at day 27 for urinalysis. The right femur of each rat was radiographed with Softex soft X-ray apparatus

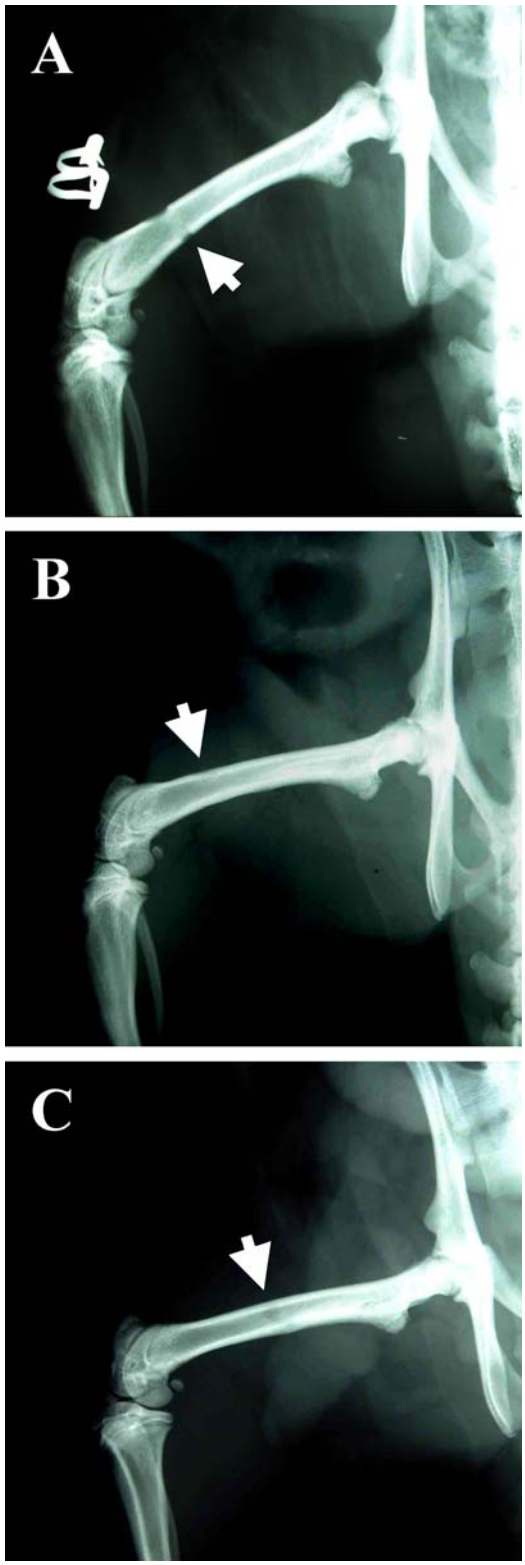

Fig. 1 Radiographs of the male rat femur implanted with β-TCP/CM-chitin at day 1 (A), and at day 28 (B), and of the sham operation group at day 28 (C). The β-TCP/CM-chitin was implanted into a penetrating defect (2 mm in diameter) at a dosage of approximately 4 mg/kg. Satisfactory bone healing was observed in the implanted group at 28 days, and cortical bone of the sham operation group was also repaired.

(Softex Co., Tokyo, Japan) both postoperatively and presacrifice.

Clinical observation

Each rat was clinically examined once a day. The weekly dietary intake of each animal was recorded, and body weight was measured once a day until sacrifice, using a Mettler Toledo electrical balance PE300.

Haematological and biochemical evaluation

Fresh blood was drawn into EDTA-2K tubes and citrated tubes from an abdominal aorta under ether anesthesia for the following assays.

Cell numbers of red blood cells, white blood cells, platelets, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells, haematocrit value, haemoglobin content, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and reticulocyte ratio were determined automatically using an automatic haematological analyzer (ADVIA120) (Bayer Healthcare Diagnostics, Tarrytown, NY, USA).

To analyze prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen content (FN), whole blood from each animal was drawn into tubes containing 3.13% citric acid, and centrifuged at $1{,}680 \times g$ for 13 min to obtain plasma. STA Compact equipment (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was used to determine plasma PT, APTT, and FN.

Total proteins, glucose, triglyceride, total cholesterol, phospholipids, blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, calcium and inorganic phosphorus in serum were determined automatically using a Clinical Analyzer 7170 (Hitachi High-Technologies Co., Tokyo, Japan). Sodium, potassium, and chloride were determined by means of the ion-selective electrode method using an automated electrolyte analyzer EA06R (A&T Co., Yokohama, Japan).

Serum was placed on a cellulose acetate membrane, stained with Ponceau S, and analyzed densitometrically to determine albumin, α-1 globulin, α-2 globulin, β-globulin, γ-globulin and the albumin/globulin ratio, using automated electrophoresis equipment Epalyzer (Helena Kenkyujyo, Saitama, Japan).

Serum immunoglobulin G (IgG) concentration was determined by means of an enzyme-linked immunosolvent assay (ELISA) using a Rat IgG ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX, USA) in accordance with the manufacture's instructions.

Urinalysis analysis

Urine collected from each rat for 3 h at day 27 was used to determine pH, occult blood, ketone bodies, glucose, protein, bilirubin, and urobilinogen, and these data were analyzed by a Bayer Clinitek 500 urine chemistry analyzer. The osmotic pressure of pooled 24-h urine samples collected at day 26–27 was determined by an Auto & Stat OM-6030 osmometer (Arkray Inc., Kyoto, Japan). Pooled 24-h urine samples were separated into supernatants and pellets by centrifugation at $1{,}680 \times g$ for 5 min.

Dissection

After drawing blood for analysis under ether anaesthesia, the rats were sacrificed and inspected for visible toxic damage by examination of the body, head, chest, abdomen, and pelvis. The extracted organs, including the brain, heart, lung, liver, spleen, kidney, adrenal gland, testis, and ovary, were weighed by an electronic balance PE160 (Mettler Toledo). Relative organ weights were calculated from organ weight per whole body weight on the dissection day.

Histopathological evaluation

Forty-four tissues and organs including brain, pituitary, spinal cord, sciatic nerve, eyeball with optic nerve, Harderian gland, lymph nodes, salivary gland, tongue, thyroid gland, parathyroids, trachea, thymus, heart, liver, pancreas, spleen, kidney, adrenal gland, lung with bronchus, esophagus, bladder, mammary gland, seminal vesicles, prostate, testis, epididymis, ovary, uterus, vagina, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, skin, aorta, muscle, bone marrow, sternum, and femur (right and left), were fixed with 10% formalin neutral buffer solution, embedded in paraffin, and sliced on glass. The sternum and femur were decalcified using hydrochloric acid solution K-CX (Falma Co., Tokyo, Japan) before paraffin embedding. The specimens were stained by hematoxylin-eosin and evaluated by microscopic examination.

Statistical analysis

All data including body weights, dietary intake, haematological values, blood chemical values, electrophoresis for serum proteins, IgG, urinalysis values, and the absolute and relative organ weights of each group were expressed as mean $\pm$ standard deviation ($n = 5$), and were analyzed by an F-test for significant difference between each control group and implanted group. Student's unpaired t-test or Aspin-Welch's paired t-test was employed for equal variance or unequal variance, respectively. Significant differences were determined at $p < 0.05$ for the F-test and t-test. The

histopathological data were also statistically analyzed using Fisher's exact probability test.

Results

Radiographic findings at surgical sites

Figure 1 shows typical radiographic images of the operation site on the male rat femur at day 1 and day 28. Good bone healing, resembling normal bone was observed at day 28 at the site implanted with β-TCP/CM-chitin (Fig. 1B), compared with the site at day 1 (Fig. 1A). The operation site of the sham control was quite well repaired at day 28 (Fig. 1C)—the diameter of the defect was not too great for remodeling. Similar findings were observed in female rats (data not shown).

Clinical symptoms

There were no obvious clinical signs or mortality related to the effects of implantation of β-TCP/CM-chitin in any of the male or female rats. Although body weight of the female implanted group at the third week was transiently significantly lower than that of the control group, body weight gain during the study did not differ between the control and implanted groups (Fig. 2). Thus, it was concluded that this finding was not due to toxicity caused by β-TCP/CM-chitin. There was no significant difference in food consumption

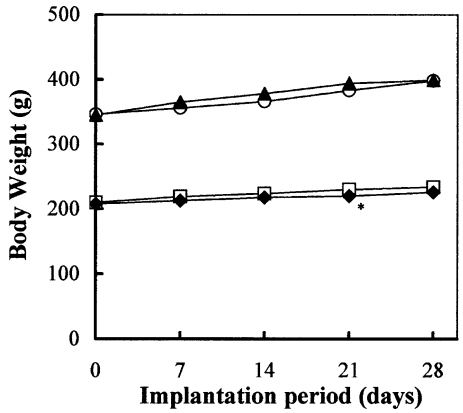

Fig. 2 Body weight change of rats with β-TCP/CM-chitin implanted in the femur for 28 days. The β-TCP/CM-chitin was implanted into a penetrating defect (2 mm in diameter) at a dosage of approximately 4 or 7 mg/kg in male (▲) and female rats (♦), respectively. A sham operation group was prepared as a control for each male (○) and female (□) rat. * Significant difference from the control group ($p < 0.05$).

Table 1 Haematological examination of rats with β-TCP/CM-chitin implanted in the femur for 4 weeks

	Male		Female	
	Control groups (Sham operation)	β-TCP/CM-chitin implanted groups	Control groups (Sham operation)	β-TCP/CM-chitin implanted groups
Number of animals	5	5	5	5
RBC ($\times 10^4$/mm^3)	853 ± 30	849 ± 27	810 ± 24	788 ± 46
Haemoglobin (g/dL)	15.5 ± 0.3	$15.9 \pm 0.2^*$	15.6 ± 0.5	15.5 ± 0.4
Haematocrit (%)	42.9 ± 0.8	43.2 ± 1.1	42.9 ± 1.4	$41.5 \pm 0.6^*$
MCV (μm^3)	50.3 ± 1.1	50.9 ± 1.3	52.9 ± 1.6	52.7 ± 2.7
MCH (pg)	18.1 ± 0.5	18.7 ± 0.6	19.2 ± 0.6	19.7 ± 1.2
MCHC (%)	36.1 ± 0.7	36.8 ± 0.6	36.4 ± 1.0	$37.4 \pm 0.6^*$
Reticulocyte (%)	2.7 ± 0.3	2.4 ± 0.3	2.7 ± 0.4	2.3 ± 0.2
Platelet ($\times 10^4$/mm^3)	90.5 ± 12.6	91.8 ± 9.4	95.5 ± 10.4	98.6 ± 17.9
WBC ($\times 10^2$/mm^3)	55.5 ± 12.8	60.4 ± 7.9	38.2 ± 14.3	35.2 ± 9.3
Differential leukocyte (%)				
Lymphocyte	77.8 ± 2.6	76.8 ± 6.1	79.7 ± 1.4	77.8 ± 2.6
Neutrophil	18.8 ± 2.1	19.4 ± 6.1	16.5 ± 1.1	18.7 ± 3.1
Eosinophil	1.2 ± 0.6	1.3 ± 0.4	1.3 ± 0.4	1.5 ± 0.9
Basophil	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Monocyte	1.5 ± 0.2	1.8 ± 0.2	1.6 ± 0.5	1.4 ± 0.6
Others (Large unstained cells)	0.7 ± 0.4	0.6 ± 0.3	0.8 ± 0.5	0.5 ± 0.2
PT (sec.)	20.0 ± 1.2	18.1 ± 0.9	16.1 ± 0.4	16.3 ± 0.5
APTT (sec.)	22.6 ± 0.7	23.3 ± 1.8	22.2 ± 1.2	21.6 ± 3.3
Fibrinogen (mg/dL)	266 ± 19	278 ± 30	237 ± 46	236 ± 16

The β-TCP/CM-chitin was implanted into the right femur of male and female rats at dosages of approximately 4 and 7 mg/kg, respectively.
RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; WBC, white blood cell; PT, prothrombin time; APTT, activated partial thromboplastin time.
Values are expressed as mean ± S.D.
* Significant difference from the control group ($p < 0.05$).

Table 2 Blood chemistry examination of rats with β-TCP/CM-chitin implanted in the femur for 4 weeks

	Male		Female	
	Control groups (Sham operation)	β-TCP/CM-chitin implanted groups	Control groups (Sham operation)	β-TCP/CM-chitin implanted groups
Number of animals	5	5	5	5
AST (U/L)	63 ± 6	65 ± 7	58 ± 6	63 ± 9
ALT (U/L)	27 ± 2	26 ± 6	18 ± 2	20 ± 3
ALP (U/L)	301 ± 30	300 ± 54	141 ± 24	120 ± 34
Total protein (g/dL)	5.99 ± 0.10	6.07 ± 0.14	6.54 ± 0.20	6.42 ± 0.29
Albumin (g/dL)	2.69 ± 0.05	2.70 ± 0.06	3.04 ± 0.10	3.08 ± 0.27
A/G	0.82 ± 0.05	0.80 ± 0.03	0.87 ± 0.04	0.92 ± 0.09
IgG (mg/ml)	15.6 ± 9.0	19.1 ± 9.5	19.1 ± 10.3	21.1 ± 7.0
Total cholesterol (mg/dL)	66 ± 11	58 ± 9	58 ± 15	48 ± 15
Triglyceride (mg/dL)	65.7 ± 18.2	79.7 ± 14.6	11.7 ± 3.9	11.8 ± 1.7
Phospholipids (mg/dL)	118 ± 14	110 ± 11	116 ± 19	103 ± 26
Glucose (mg/dL)	149 ± 21	156 ± 15	114 ± 9	116 ± 21
BUN (mg/dL)	15.7 ± 1.4	16.9 ± 2.3	21.7 ± 3.4	20.5 ± 3.5
Creatinine (mg/dL)	0.26 ± 0.03	0.30 ± 0.05	0.37 ± 0.06	0.34 ± 0.12
Na (mmol/L)	144.5 ± 1.8	143.0 ± 1.0	143.1 ± 1.1	142.8 ± 0.9
K (mmol/L)	4.25 ± 0.18	4.32 ± 0.40	3.79 ± 0.37	4.19 ± 0.45
Cl (mmol/L)	110.1 ± 2.1	108.4 ± 1.3	110.2 ± 1.9	109.0 ± 1.3
Ca (mg/dL)	9.93 ± 0.45	9.90 ± 0.18	9.92 ± 0.23	10.10 ± 0.28
IP (mg/dL)	6.38 ± 0.80	5.83 ± 0.77	6.25 ± 1.06	6.06 ± 0.49

The β-TCP/CM-chitin was implanted into the right femur of male and female rats at dosages of approximately 4 and 7 mg/kg, respectively.
AST, aspartic aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; A/G, albumin/globulin ratio; IgG, immunoglobulin G; BUN, blood urea nitrogen; IP, inorganic phosphorus.
Values are expressed as mean ± S.D.
* Significant difference from the control group ($p < 0.05$).

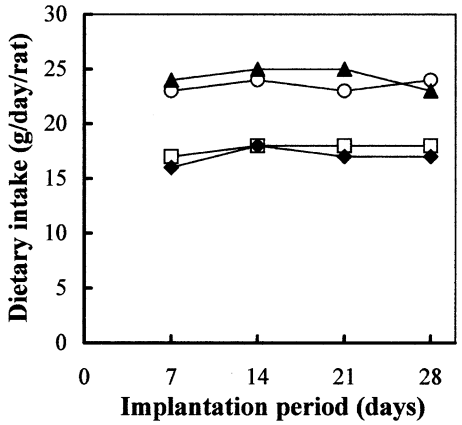

Fig. 3 Dietary intake of rats with β-TCP/CM-chitin implanted in the femur for 28 days. The β-TCP/CM-chitin was implanted into a penetrating defect (2 mm in diameter) at a dosage of approximately 4 or 7 mg/kg in male (▲) and female rats (♦), respectively. A sham operation group was prepared as a control for each male (○) and female (□) rat.
* Significant difference from the control group ($p < 0.05$).

between the control and implanted groups of either sex (Fig. 3).

Haematology and blood chemistry

Data for the haematology and blood chemistry of rats with or without implanted β-TCP/CM-chitin are shown in Tables 1 and 2, respectively. In male rats, a statistically significant difference was detected in haemoglobin values, but this difference was slight and acceptable. A significant decrease in haematocrit and an increase in MCHC were observed within the female implantation group as compared with the control group, but no difference in RBC was detected between these groups. These changes were not considered to be related to β-TCP/CM-chitin implantation. There were no significant differences between the control and implanted groups in any evaluated blood chemistry parameters.

Urinalysis

Table 3 summarizes urinary data for rats implanted with composite for 4 weeks. None of the urinalysis parameters evaluated in the implanted group of either sex differed significantly from the control group.

Organ weights

The absolute and relative organ weights of test animals are shown in Table 4. A significant decrease in the absolute weight of the liver, but not in the relative weight of the liver, was found in the male implanted group. A significant decrease in the relative weight of the kidneys, but not in the absolute weight of the kidneys, was found in the female

Table 3 Urinalysis of rats with β-TCP/CM-chitin implanted in the femur for 4 weeks

		Male		Female	
		Control groups (Sham operation)	β-TCP/CM-chitin implanted groups	Control groups (Sham operation)	β-TCP/CM-chitin implanted groups
Number of animals		5	5	5	5
Volume (mL)		16.0 ± 4.7	20.4 ± 8.8	14.4 ± 5.2	14.9 ± 4.4
Osmolality (mOsm/kg)		1297 ± 607	869 ± 305	1196 ± 332	1125 ± 209
Na (mmol/day)		1.40 ± 0.44	1.02 ± 0.40	1.32 ± 0.23	1.31 ± 0.23
K (mmol/day)		2.64 ± 0.62	2.19 ± 0.80	2.26 ± 0.39	2.27 ± 0.49
Cl (mmol/day)		1.48 ± 0.54	1.06 ± 0.59	1.48 ± 0.26	1.50 ± 0.36
Color	Slight yellow	5	5	5	5
pH	6.5	1	–	–	–
	7.0	–	–	1	1
	7.5	–	–	–	–
	8.0	2	1	1	4
	8.5	2	4	3	–
Occult blood	–	3	2	3	4
	–/+	2	3	–	1
	1+	–	–	2	–
Ketone bodies	–	–	–	4	4
	–/+	3	3	1	1
	1+	2	2	–	–
Glucose	–	5	5	5	5
Protein	–	–	–	1	3
	–/+	–	1	1	1
	1+	4	3	2	1
	2+	1	1	1	–
Bilirubin	–	5	5	4	5
	1+	–	–	1	–
Urobilinogen	–/+	3	4	3	4
	1+	2	1	1	1
	2+	–	–	1	–
Urinary sediment					
Erythrocytes	–	5	5	5	5
Leukocytes	–	5	5	5	5
Epitherial cells	–	5	5	5	5
Casts	–	5	5	5	5
Far globule	–	5	5	5	5
Mucous threads	–	5	5	5	5
Others	–	–	1	–	–
	+	5	4	5	5

The β-TCP/CM-chitin was implanted into the right femur of male and female rats at dosages of approximately 4 and 7 mg/kg, respectively.
Values are expressed as mean ± S.D.
* Significant difference from the control group ($p < 0.05$).

implanted group. These changes were not considered to be related to toxicity of the implanted composite.

Histopathological evaluation

Table 5 summarizes the histopathological findings of rats implanted with the composite for 28 days. No significant differences in the findings specifically caused by β-TCP/CM-chitin implantation were found in either sex. The tissue response at the remodeling bone site in both the implanted and control groups was a normal reaction, except for the finding of a few residual β-TCP particles derived from the composite in the experimental groups, especially in females. The following histopathological findings were thought to be related to surgical trauma or to the administration of anaesthetic: mononuclear cells in the heart, microgranuloma and accumulation of foamy cells in the lung, erosion in the glandular stomach, acinar atrophy in the pancreas, degeneration of vascular sinusoidal cells, fatty changes of hepatocyte, microgranuloma, polyarteritis, capsular thickening and scarring in the liver, basophilic tubules, dilatation of tubules, hyaline droplets, mineralization and dilation of the renal pelvis,

Table 4 Absolute and relative organ weights of rats with β-TCP/CM-chitin implanted in the femur for 4 weeks

	Male		Female	
	Control groups (Sham operation)	β-TCP/CM-chitin implanted groups	Control groups (Sham operation)	β-TCP/CM-chitin implanted groups
Number of animals	5	5	5	5
Body weight (g)	371 ± 31	374 ± 20	217 ± 7	210 ± 5
Absolute organ weight				
Brain (g)	2.12 ± 0.08	2.12 ± 0.11	1.98 ± 0.07	1.94 ± 0.07
Heart (g)	1.05 ± 0.07	1.06 ± 0.04	0.71 ± 0.03	0.70 ± 0.08
Lungs (g)	1.19 ± 0.05	1.21 ± 0.07	0.92 ± 0.05	0.87 ± 0.04
Liver (g)	9.87 ± 1.27	9.69 ± 0.89	5.99 ± 0.38	$5.57 \pm 0.28^{*}$
Kidneys (g)	2.46 ± 0.32	2.31 ± 0.21	1.59 ± 0.07	1.59 ± 0.04
Spleen (g)	0.68 ± 0.08	0.70 ± 0.09	0.44 ± 0.04	0.45 ± 0.07
Adrenal glands (mg)	75 ± 10	82 ± 11	102 ± 14	100 ± 11
Testes (g)	3.44 ± 0.14	3.02 ± 0.96	–	–
Ovaries (g)	–	–	108 ± 27	113 ± 28
Relative organ weight				
Brain (%)	0.574 ± 0.054	0.570 ± 0.057	0.913 ± 0.042	0.926 ± 0.029
Heart (%)	0.283 ± 0.022	0.284 ± 0.010	0.328 ± 0.010	0.331 ± 0.032
Lungs (%)	0.322 ± 0.018	0.324 ± 0.029	0.425 ± 0.023	0.414 ± 0.019
Liver (%)	2.656 ± 0.162	2.584 ± 0.105	2.763 ± 0.161	2.652 ± 0.151
Kidneys (%)	0.663 ± 0.040	$0.618 \pm 0.031^{*}$	0.735 ± 0.042	0.757 ± 0.028
Spleen (%)	0.183 ± 0.013	0.186 ± 0.020	0.204 ± 0.020	0.216 ± 0.033
Adrenal glands (%)	0.020 ± 0.003	0.022 ± 0.002	0.047 ± 0.007	0.048 ± 0.004
Testes (%)	0.933 ± 0.098	0.803 ± 0.249	–	–
Ovaries (%)	–	–	0.050 ± 0.013	0.054 ± 0.013

The β-TCP/CM-chitin was implanted into the right femur of male and female rats at dosages of approximately 4 and 7 mg/kg, respectively. Values are expressed as mean $\pm$ S.D.

*Significant difference from the control group ($p < 0.05$).

Table 5 Summary of histopathological findings of rats with β-TCP/CM-chitin implanted in the femur for 4 weeks

	Male							Female						
	Control groups (Sham operation)				β-TCP/CM-chitin implanted groups			Control groups (Sham operation)				β-TCP/CM-chitin implanted groups		
Findings in organs		1	2	3		1	2	3		1	2	3		1	2	3
Number of animals necropsied			5				5				5				5	
Heart	(5)				(5)				(5)				(5)			
cellular infiltration, mononuclear		1	0	0		1	0	0		0	0	0		0	0	0
Lung	(5)				(5)				(5)							
accumulation of foamy cells		2	0	0		1	0	0		0	0	0		0	0	0
microgranuloma		0	0	0		0	0	0		1	0	0		0	0	0
Grandular stomach	(5)				(5)				(5)				(5)			
erosion		0	0	0		0	0	0		0	0	0		1	0	0
Exocrine pancreas	(5)				(5)				(5)							
atrophy, acinus		0	0	0		1	0	0		0	0	0		1	0	0
Liver	(5)				(5)				(5)				(5)			
degeneration, vacuolar, sinusoidal cells		0	0	0		0	0	0		0	0	0		1	0	0
fatty change, hepatocyte		0	0	0		1	0	0		3	0	0		0	0	0
microgranuloma		4	0	0		4	0	0		1	0	0		2	0	0
polyarteristis		0	0	0		1	0	0		0	0	0		0	0	0
capsular thickening		3	0	0		3	0	0		4	0	0		3	0	0
scar		0	0	0		0	0	0		1	0	0		0	0	0

Continued

Table 5 Continued

	Male								Female							
	Control groups (Sham operation)				β-TCP/CM-chitin implanted groups				Control groups (Sham operation)				β-TCP/CM-chitin implanted groups			
Findings in organs		1	2	3		1	2	3		1	2	3		1	2	3
Kidney	(5)				(5)				(5)				(5)			
basophilic tubules		1	0	0		0	1	0		3	0	0		5	0	0
dilatation, tubules		0	0	0		0	0	0		1	0	0		1	0	0
hyaline droplet		2	0	0		1	0	0		0	0	0		0	0	0
mineralization		0	0	0		1	0	0		5	0	0		5	0	0
dilatation, renal pelvis		1	0	0		2	0	0		1	0	0		0	0	0
Urinary bladder	(5)				(5)				(5)				(5)			
cellular infiltratation, lymphocyte		1	0	0		0	0	0		0	0	0		0	0	0
Testis	(5)				(5)				(–)				(–)			
atrophy, seminiferous tubule		2	0	0		1	0	1		–	–	–		–	–	–
mineralization		0	0	0		1	0	0		–	–	–		–	–	–
Epididymis	(5)				(5)				(–)				(–)			
no sperm		0	0	0		1	0	0		–	–	–		–	–	–
Prostate	(5)				(5)				(–)				(–)			
cell debris, lumen		4	0	0		5	0	0		–	–	–		–	–	–
cellular infiltration, lymphocyte		5	0	0		2	0	0		–	–	–		–	–	–
Uterus	(5)				(5)				(5)				(5)			
hemorrhage		–	–	–		–	–	–		0	0	0		1	0	0
dilatation, lumen		–	–	–		–	–	–		2	0	0		1	0	0
Broad ligament of uterus	(5)				(5)				(5)				(5)			
adhesion		–	–	–		–	–	–		–	–	–		1	0	0
Pituitary gland	(5)				(5)				(5)				(5)			
cyst		0	0	0		1	0	0		0	0	0		0	0	0
Thyroid gland	(5)				(5)				(5)				(5)			
ultimobranchial remnant		0	0	0		0	0	0		0	0	0		1	0	0
Treatment site (right femur)	(5)				(5)				(5)				(5)			
hemorrhage		1	0	0		0	0	0		0	0	0		0	0	0
degeneration, muscle fiber		0	0	0		1	0	0		0	0	0		0	0	0
foreign body (residual material)		0	0	0		3	0	0		0	0	0		5	0	0
mineralization, periosteum		1	0	0		1	0	0		0	0	0		0	0	0
foreign body reaction		0	0	0		1	0	0		0	0	0		1	0	0
fibrosis		0	0	0		1	0	0		0	0	0		0	0	0
remodeling, bone		2	0	0		5	0	0		3	0	0		4	0	0

1: Slight 2: Moderate 3: Marked, –: Not applicable.
The β-TCP/CM-chitin was implanted into the right femur of male and female rats at dosages of approximately 4 and 7 mg/kg, respectively.
Numbers in paranthesis indicate No. of animals examined.
* Significant difference from the control group ($p < 0.05$).

lymphocytic cellular infiltration of the urinary bladder, atrophy of the seminiferous tubules, mineralization of the testis, aspermia in the epididymis, cell debris in the lumen and lymphocytic cellular infiltration of the prostate, hemorrhage and dilation of the lumen of the uterus, adhesion of the broad ligament of the uterus, pituitary gland cyst, or an ultimobranchial remnant in the thyroid gland.

Discussion

Biocompatibility as well as functional efficacy in meeting clinical objectives are essential characteristics for biomaterials, and the International Organization for Standardization (ISO10993) specifies various biological evaluations under the Good Laboratory Practice standard for

medical devices and materials prior to clinical trials. The β-TCP/CM-chitin composite was developed as a bone filler and was designed to be biodegradable within a month and replaced by host-derived bone tissue. According to the categorization described in ISO10993-1, at least, cytotoxicity, sensitizasion, irritation or intracutaneous reactivity, systemic toxicity, subacute toxicity, genotoxicity, and implantation were essential initial evaluation tests for assessment of β-TCP/CM-chitin. In addition, carcinogenicity and biodegradation tests were also required for bioabsorbable bone fillers. Chemical characterization and carcinogenic potency of the components (extracts) of β-TCP/CM-chitin had already been investigated by *in vitro* assays to confirm their biocompatibility. These assays included the Ames test, chromosome aberration assay, cell transformation assay, and metabolic cooperation assay [7].

In the present study, to further assess the biological safety of β-TCP/CM-chitin as a bone filler, the systemic toxicity of the composite implanted into the femur was evaluated using SPF rats during a term (28 days) approximately corresponding to its in situ degradation time. The protocol of this test was designed to elucidate systemic toxicity, subacute toxicity, and implantation tests in accordance with ISO10993. The maximal bone defect size in rat femur was determined to be 2 mm in diameter to avoid fractures and subsequent non-specific changes—this size would not prevent healing of a bone defect. β-TCP/CM-chitin was implanted at a dosage of approximately 4 mg/kg and 7 mg/kg in male and female rats, respectively. Soft X-ray radiographs showed that sufficient bone density of cortex bone was acquired in the male implanted group by day 28 (Fig. 1B), whereas discontinuity of bone cortex was clearly seen on post-operation day 1 (Fig. 1A). Bone healing of the defect in the sham operation group was also adequate at 4 weeks (Fig. 1C). These findings were common to males and females.

No characteristic changes indicating significant systemic toxicity caused by β-TCP/CM-chitin implantation were found in any male and female rats in the present study, as shown in Tables 1–4. Histopathological findings also support the safety of the implanted material (Table 5). It is known that CM-chitin, a novel biomaterial, is first hydrolyzed to an oligomer by endogenous lysozymes secreted by recruited neutrophils and macrophages during inflammation [10], and that intravascular CM-chitin is excreted as urea [11]. The high biodegradability by lysozymic hydrolysis [9, 12] and the low immunotoxicity [13–15] of CM-chitin make it suitable for application as a bioabsorbable material in terms of biological safety. The toxicity of the CM-chitin component was thought to be quite low, since the CM-chitin content of the composite was only about 16.7% (wt/wt), and the biodegraded CM-chitin component was mainly extracted as urea via body fluids and blood. Thus, the systemic toxicological behavior of β-TCP/CM-chitin was thought to be similar to β-TCP alone. The biological safety and efficacy of β-TCP was also confirmed by the accumulated clinical evidence.

In conclusion, no subacute systemic toxicity of β-TCP/CM-chitin implanted into the femur was found at the dosage of 4 or 7 mg/kg in male and female rat models, respectively. Therefore, β-TCP/CM-chitin is expected to be a highly biocompatible bone substitute, at least at these dosages.

Acknowledgment The authors thank the members of Experimental Toxicology Department (Biosafety Research Center, Foods, Drugs and Pesticides) for their technical assistance.

References

1. S. N. BHASKAR, J. M. BRADY, L. GETTER, M. F. GROWER and T. DRISKELL, Biodegradable ceramic implants in bone. Electron and light microscopic analysis. *Oral. Surg. Oral. Med. Oral. Pathol.* **32** (1971) 336–346.
2. D. E. CUTRIGHT, S. N. BHASKAR, J. M. BRADY, L. GETTER and W. R. POSEY, Reaction of bone to tricalcium phosphate ceramic pellets. *Oral. Surg. Oral. Med. Oral. Pathol.* **33** (1972) 850–856.
3. H. U. CAMERON, I. MACNAB and R. M. PILLIAR, Evaluation of biodegradable ceramic. *J. Biomed. Mater. Res.* **11** (1977) 179–186.
4. E. B. NERY and K. L. LYNCH, Preliminary clinical studies of bioceramic in periodontal osseous defects. *J. Periodontal.* **49** (1978) 523–527.
5. S. MASUDA, Y. YOSHIHARA, K. MURAMATSU and I. WAKEBE, Repairing of osteochondral defects in joint using beta-TCP/carboxymethyl chitin composite. in "Key Engineering Materials Vol. 284–286 (2005) (Bioceramics Vol. 17). Proceedings of the 17th International Symposium on Ceramics in Medicine," edited by P. LI, K. ZHANG and C. W. COLWELL (New Orleans, 2004) p. 791–794.
6. K. MURAMATSU, S. MASUDA, Y. YOSHIHARA and A. FUJISAWA, In vitro degradation behavior of freeze-dried carboxymethyl-chitin sponges processed by vacuum-heating and gamma irradiation. *Polym. Degrad. Stab.* **81** (2003) 327–332.
7. K. MURAMATSU, M. NAKAJIMA, M. KIKUCHI, S. SHIMADA, K. SASAKI, S. MASUDA and Y. YOSHIHARA, In vitro cytocompatibility assessment of β-tricalcium phosphate/carboxymethyl-chitin composite. *J. Biomed. Mater. Res.* **71A** (2004) 635–643.
8. I. MATSUMURA and J. F. KIRSCH, Is aspartate 52 essential for catalysis by chicken egg white lysozyme? The role of natural substrate-assisted hydrolysis. *Biochemistry* **35** (1996) 1881–1889.
9. A. C. A. WAN, E. KHOR, J. M. WONG and G. W. HASTING, Promotion of calcification on carboxymethylchitin discs. *Biomaterials* **17** (1996) 1529–1534.
10. R. A. A. MUZZARELLI, Human enzymatic activities related to the therapeutic administration of chitin derivatives. *Cellular and Molecular Life Science* **53** (1997) 131–140.
11. H. OHNISHI, Y. KATO, H. HATA and Y. MACHIDA, Biological fates of N-succinylchitosan and 6-O-carboxymethylchitin after intravenous injection in mice. in "Chitin and Chitosan:

Chitin and chitosan in life science," edited by T. URAGAMI, K. KURITA and T. FUKAMIZO (Kodansha Scientific, Tokyo, 2001) p. 206–209.
12. I. MATSUMURA and J. F. KIRSCH, Is aspartate 52 essential for catalysis by chicken egg white lysozyme? The role of natural substrate-assisted hydrolysis. *Biochemistry* **35** (1996) 1881–1889.
13. K. NISHIMURA, S. NISHIMURA, N. NISHI, I. SAIKI, S. TOKURA and I. AZUMA, Immunological activity of chitin and its derivatives. *Vaccine* **2** (1984) 93–99.
14. K. NISHIMURA, S. NISHIMURA, N. NISHI, F. NUMATA, Y. TONE, S. TOKURA and I. AZUMA, Adjuvant activity of chitin derivatives in mice and guinea-pigs. *Vaccine* **3** (1985) 379–384.
15. A. YOKOYAMA, H. MATSUNO, S. YAMAMOTO, T. KAWASAKI, T. KOHGO, M. UO, F. WATARI and M. NAKASU, Tissue response to a newly developed calcium phosphate cement containing succinic acid and carboxymethyl-chitin. *J. Biomed. Mater. Res.* **64A** (2003) 491–501.