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Abstract

Safety Evaluation of Nanocalcium Based on Physicochemical Characteristics

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This study was performed to assess the potential toxicity of nanocalcium in terms of physico-chemical characteristics. After the blind test of seven samples of calcium provided from Korea Food and Drug Administration using energy-filtering transmission electron microscopy (EF-TEM), componential analysis using X-ray diffractometry (XRD), and energy dispersive spectrometry, two samples of nano and micro sized calcium were selected for further study. The characteristics of nano and microcalcium were evaluated by EF-TEM, field-emission scanning electron microscopy, XRD, dynamic light scattering, and electron spin resonance. Both samples were agglomerated and did not produce any measurable reactive oxygen species. The specific surface area of nano- and microcalcium measured by the N2 Brunauere Emmette Teller (BET) method was 12.90 ± 0.27 m^2/g, and 1.12 ± 0.19 m^2/g,

respectively. Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis revealed the release of 2–3 times more calcium ion from nanocalcium compared to microcalcium at pH 5 and 7. Genotoxicity and single-dose and repeated-dose 14-days oral toxicity testing in Sprague-Dawley rats were performed to evaluate the safety of nanocalcium. The results of BET and ICP-OES analysis showed that nanocalcium has a large specific surface area and a greater potential for ionization. Neither genotoxicity nor in vivo toxicity was observed in our experimental conditions. However, we suggest long-term monitoring is need for nanocalcium due to their differential of ionization derived from their different specific surface area.

Keywords: nanocalcium, genotoxicity, physico-chemical characteristics, specific surface area

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LIST OF ABBREVIATION

ADME	Adsorption, Distribution, Metabolism, Excretion
A/G	Albumin / Globulin
ALB	Albumin
ALP	alkaline phosphatase
ALT	alanine transaminase
AST	aspartate transaminase
BET	Brunauer–Emmett–Teller
BUN	blood urea nitrogen
DLS	Dynamic light scattering
Ca	calcium
CHO-k1	Chinese hamster ovary fibroblasts cells
Cl	chloride
DMPO	5,5-Dimethyl-1-pyrroline N-oxide
EF-TEM	Energy-filtering transmission electron microscope
ESR	Electron spin resonance
FBS	Fetal bovine serum
FE-SEM	Field emission scanning electron microscopy
GGT	Gamma(γ)-glutamyl transferase
GI50	growth inhibition 50%
Hb	hemoglobin
HCT	Hematocrit
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
IP	Inorganic phosphorus
K	potassium
LD_{50}	Lethal dose 50
LDH	lactate dehydrogenase
LUC	Large unstained cells
MCH	Mean corpuscular hemoglobin

MCHC	Mean corpuscular hemoglobin conc.
MCV	Mean corpuscular volume
Mg	magnesium
MNPCEs	Micronucleated polychromatic erythrocytes
MPV	Mean Platelet Volume
Na	sodium
NCEs	Normochromatic erythrocytes
PCEs	Polychromatic erythrocytes
PLT	Platelet
RBC	red blood cells
RDW	Red cell distribution width
ROS	Reactive oxygen species
SD	Sprague-Dawley
SPF	Specific pathogen free
WBC	white blood cells
XRD	X-ray diffractometry

INTRODUCTION

1) Application of nanotechnology

Scientific technology of 21st century is considered to be led by nanotechnology. Nanotechnology is driving the creation of a myriad of novel materials with many applications in disciplines as diverse as medicine, electronics, biomaterials and the food industry. Applications of nanotechnology make revolution in the food industry including improvement of taste, flavor, color, safety and food packaging. Global market value of nanofood applications including food packaging was estimated at US$4 million in 2006, predicted to range between US$6 billion by 2012(Cientifica,2006) and more than 20 billion by 2010 (Helmut Kaiser Consultancy, 2004).

Reducing the particle size of materials may be an efficient and reliable tool for improving the bioavailability and absorption in food. Indeed, nanonization of the particles improved absorption rate of drug or nutrients in human (Liversidge and Cundy 1995; Merisko-Liversidge and Cooper 2003; Douroumis and Fahr 2006). Since the application of nanotechnology to health food science to stabilize bioactive materials and improve their bioavailability (Mozafari et al., 2008), various trials had sought to enhance the capabilities of nanotechnology in food, for example, in overcoming the limitations of absorption efficiency of specific essential components. Coming into the market nanoparticulate additives or supplements such as calcium, magnesium, iron, zinc, omega-3 fatty acid, lycopene, Vitamin D2 and beta-carotene

shows application of nanotechnology for healthfood.

2) Efficacy of nanocalcium

Calcium is essential for living organisms, in particular for the cell physiology, where calcium influx and efflux functions as a signal for many cellular processes. As a major material used in mineralization of bone, teeth, calcium is the most abundant metal by mass in many animals. Calcium deficiency leads to osteoporosis.

Despite its physiological significance, the absorption efficiency of calcium is difficult to enhance. Various studies have sought to improve the absorption efficiency. Some recent study showed nanocalcium improved absorption efficiency and bioavailability. Park et al., (2008) compared the effect of nanocalcium to other calcium form and reported that consumption of nanocalcium enriched milk resulted in increasing the urinary excretion of calcium, while decreasing that of deoxypyridinoline and hydroxyproline in ovary ectomized (OVX) rats. The study suggests that nano-calcium enriched milk effectively promotes bone formation in OVX rats. Huang et al (2009) showed that nanocalcium carbonate and nanocalcium citrate is more bioavailable than microcalcium carbonate and microcalcium citrate, respectively. Nano-sized calcium is expected to be easily absorbed into the body due to the large surface area (Hu et al., 2010). Also, Chen et al.,(2008) showed nanonized peal powder improves bioavailability and absorption of calcium compare with micronized peal powder. The collective data have demonstrated that nanocalcium is more effective by virtue of the increased of the surface area.

3) Potential toxicological effect of nanomaterials

Whereas potential benefits of nanoproducts are emphasized, potential toxicological effects of nanomaterials are little known. Nanoparticles may appear toxicological effect on biological system even if bulk materials of the same composition dose not shown toxicity (Nel et al., 2006; Oberdorster et al., 2005). Due to increased surface area, nanomaterials have highly reactive characteristic which potentially could lead to toxicity (Oberdorster et al., 2005). Applerot et al. (2009) and Hanley et al. (2009) showed ZnO nanomaterials are more toxic than macro-sized ZnO particles. Chen et al. (2006a, 2006b) determined the acute toxicity of copper particles and nanocopper in mice and found that nanocopper was more toxic than bulk copper. Used nano-sized particles for food may have different properties from macro-sized particles that influence on our bodies.

4) Need for safety evaluation of nanocalcium

Toxicologically, it has been premature to conclude that nanocalcium is relatively more toxic than larger-sized calcium. Huang et al. (2009) estimated calcium carbonate and calcium citrate in both the nano and micro form in vitro and in vivo using ICR mice. But, for calcium carbonate, the concentration of high dose group was insufficient to assess toxicity. In OECD Test Guideline, the concentration of the high dose group was recommended as 2,000 mg/kg. Hu et al. (2010) showed that polyacrylic acid (PAA) can provide a more biologically friendly pathway to prepare biological nanocalcium phosphate for biomedical applications by assessing the viability of bone marrow mesenchymal stem cells (BMSCs) in vitro. In this report, in vivo toxicity was not assessed, not was the effect on normal cell lines. Park et al. (2008) also did not

provide sufficient data to draw any conclusion about the toxicity of nanocalcium. Genotoxicity and in vivo toxicity test data are lacking, but are essential to estimate the toxicity of nanocalcium.

Also, it is important to estimate the toxicity of nanocalcium based on physico-chemical properties. In general, nanomaterials including nanocalcium have several attributes: (1) nanoparticles are more chemically reactive than larger particles because of their increased surface area; (2) nanoparticles have greater access to our bodies than larger particles; (3) the enhanced bioavailability and bioactivity of nanoparticles may introduce new and unknown toxicity; (4) nanoparticles may be capable of disrupting immune responses; and (5) nanoparticles may have long-term pathological effects because of their low excretion rate (Oberdörster et al., 2005; Nel et al., 2006; Chithrani et al., 2006; Sonavane et al., 2008). In this study, nano- and microcalcium were compared in terms of their physico-chemical properties to estimate the genotoxicity and in vivo toxicity of nanocalcium. The goal was to productively contribute to determining the maximum concentration of nanocalcium in a 13-week oral repeated dose toxicity test.

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MATERIALS AND METHODS

Blind test and selection of nanocalcium and microcalcium

After the blind test for seven samples provided from Korea Food and Drug Administration (KFDA), we selected nanocalcium and microcalcium based on their size and purity. Both type of particles were provided at >98.0% purity. The powders were stored at room temperature until analysis.

Characterization of nano- and microcalcium

Measurement of size of nanocalcium and microcalcium

High resolution images were obtained by energy-filtering transmission electron microscopy (EF-TEM) using a LIBLA 120 microscope (Carl Zeiss, Oberkochem, Germany) at an accelerating voltage of 120 kV. A dilute suspension (4 mg/ml) of particles was prepared in methanol and sonicated for 30 s. A portion of the aqueous dispersion was deposited on a 300-mesh carbon-coated copper grid and dried at room temperature overnight before examination. To demonstrate the morphology and size of the particles, the samples were examined by field-emission scanning electron microscopy (FE-SEM) using a SUPRA 55VP instrument (Carl Zeiss) operating at 15 kV. For FE-SEM, samples were placed on conductive copper tape and analyzed. The

electrical conductivity of the samples was enhanced by coating them with platinum via sputtering (BAL-TEC, Witten/Ruhr, Germany) before examination.

X-ray diffractometry (XRD)

XRD was conducted using a D8 Advence (Bruker, Rheinstetten, Germany) investigate the crystal structure of nano- and microcalcium particles. Samples of about 1 g in weight were placed onto an aluminum holder. The goniometer was motorized and moved through a range of θ–2θ mode scan. The diffractometer was run at 40 kV and 40 mA in the range of 20–80 °. Steps were increments of 0.02 °, and counts were collected for 2 s at each step.

Dynamic light scattering (DLS) measurements

Particle size and distribution of nanocalcium and microcalcium were determined using an ELS-8000 electrophoretic light scattering spectrophotometer (Photal, Osaka, Japan) based on the DLS principle. For these measurements, 4 ml of a 0.2 mg/ml suspension of particles in methanol was ultra-sonicated for 30 s. To determine particle size and distribution, each particle suspension was measured at 30 the number of circuits at room temperature.

Measurement of specific surface area

Specific surface area of nanocalcium and microcalcium particles was determined by the N2 Brunauere Emmette Teller (BET) method using a BELSORP-max (MP)

analyzer (BEL, Osaka, Japan). Dried and degassed samples were analyzed using a multipoint N2 adsorption-desorption method at room temperature (Streppel et al., 2011; Brunauer et al., 1938).

Electron spin resonance (ESR) spectroscopy and spin-trapping measurements

ESR signals of samples were taken using a JES-TE200 ESR spectrometer (JEOL, Tokyo, Japan) with a X-band standard frequency of 8.8–9.6 GHz. To identify the peaks, the signal components were analyzed by the ES-IPRITS DATA SYSTEM, Version 3.00 analysis software installed in the ESR instrument (ESR computer software. ESR spectra were measured for 2 min after the introduction of nano- or microcalcium. The electronic measurement conditions were: frequency 9.42 GHz, mod 100 kHz, time constant 0.03 s, and power 5.00 mW. Aqueous samples were loaded into a LC-12 aqueous quartz flat cell (JEOL). To detect •OH (hydroxyl) and $1O_2$ (singlet oxygen) radicals in a water suspension of nanocalcium and microcalcium (10 mg/ml), the ESR spin-trapping technique was designed. Spin-trap agents and scavengers used in this study were 5,5-dimethyl-1-pyrroline N-oxide (DMPO) for •OH and 2,2,6,6-tetramethylpiperidine (TEMP) for $1O_2$.

Ca^{2+} release from nano- and microcalcium particles

Inductively coupled plasma optical emission spectrometry (ICP-OES) was carried out using an ICP-730 ES apparatus (Varian, California, USA). ICP-OES is a sensitive

means of determining both the presence of metal analytes and their concentrations. To confirm the release of Ca^{2+} from nano- and microcalcium particles, the pH-dependent release of Ca^{2+} was analyzed. The concentration of nano- and microcalcium particles suspended in solution for testing was determined at 10 mg/ml and samples were measured after suspension in solution for 6 h. The pH of the solvents was 2, 5, and 7.

Genotoxicity of nanocalcium

Chromosomal aberration test

This test was performed according to OECD Test Guideline No. 473 (OECD, 1997). Chinese hamster ovary fibroblasts cells (CHO-*k*1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in F-12 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 mg/ml) at 37 ^{o}C in a humidified atmosphere of 5% CO_2. CHO-k1 cells were exposed to nanocalcium particles for a short-term (6 h) and long-term (24 h). Mitomycin C (direct method) and cyclophosphamide (metabolic activation method) were used as the positive control substance. The cytotoxicity test was expressed as growth inhibition 50% (GI50) by calculating the probit. To make chromosome slides, the harvested cultures were incubated with a 0.075 M KCl for 20 min. The cells were fixed in a methanol / glacial acetic acid solution 3 : 1 (v/v). This step was repeated three times and the cells were dropped onto a slide. After air drying, the cells were

stained with 5% Giemsa (Merck, Darmstadt, Germany) solution. At least two slides were generated per culture and 200 metaphase events were analyzed per concentration.

Micronucleus assay

This study was performed in accordance with OECD Test Guideline 474 (OECD, 1997). Seven-week-old male ICR mice were purchased from ORIENT BIO (Gyeonggi-do, Korea). Only healthy animals were selected for this study by observation of the absence of general symptoms for 7 days of the acquisition. Nanocalcium was administrated orally using a sonde. The positive control substance was administered intraperitoneally to test animals using a disposable 26G syringe. According to the preliminary study result, the highest dose was determined to 2000 mg/kg. Test groups were consisted of five groups (n=6 animals per group): negative control group (distilled water), three dose groups (500 mg/kg, 1000 mg/kg, and 2000 mg/kg), and one positive control group (Mitomycin C (MMC), 2.0 mg/kg). Bone marrow smears were done 24 h after administration by staining with acridine orange or 4% Giemsa solution. The number of micronucleated polychromatic erythrocytes (MNPCEs) per 2000 PCEs for each mouse was counted at 400-fold magnification using a fluorescence microscope (Leica, Wetzlar, Germany). The ratio of PCEs to normochromatic erythrocytes [i.e., PCE / (PCE+NCE)] was calculated based on a total of 200 erythrocytes at 1000-fold magnification (Carl Zeiss). Statistical analyses were performed using SPSS 12.0 (SPSS, Chicago, IL, USA) and the data was expressed as

the mean ± SD. One-way analysis of variance (ANOVA) was applied to the micronucleus test all the data. A value of $p < 0.05$ indicated statistical significance.

Bacterial reverse mutation assay

In this study, we used *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2uvrA (Molecular Toxicology, Boone, NC, USA). This study was conducted using a pre-incubation method in the presence and absence of metabolic activation, according to the OECD Test Guideline 471 (OECD, 1997). The test substance, nanocalcium, was supplied from KFDA and was dispersed in distilled water. The strains were cultured in Oxoid nutrient broth No. 2 for 10 h at 37 ^{o}C with shaking. After culture, strains were added to different concentration (0, 313, 625, 1250, 2500, and 5000 μg/plate) of nanocalcium, which were dispersed in 50 μl distilled water and 0.5 ml S9 mixture (or sodium phosphate buffer). They were incubated in shaking water bath at 100 rpm rotating speed and at 37 ^{o}C for 20 minutes. Two milliliters top agar were added and poured onto glucose minimal agar plate. Plates were incubated in vented incubator at 37 ^{o}C for 48 h. After that, the number of revertant colonies was counted. If there was over 2-fold increase of the negative control value or dose-dependent increase in the number of revertants in one or more strains, a decision was made on the mutagenecity. The study was performed twice (preliminary-dose finding and main study) with triplicate determinations at each dose level.

Single-dose acute oral toxicity

This test was performed in accordance with KFDA Notification No. 2009-116 (Toxicity Test Standards of Medicine and Medical Supplies, Revised August 24, 2009). The test substance, nanocalcium, was supplied from KFDA and was dispersed in distilled water. Seven-week-old male and female, specific-pathogen free (SPF) Sprague-Dawley (SD) rats supplied from ORIENT BIO were acclimated for 7 days before administration. During the acclimation and experimental periods, the rats were housed in stainless wire mesh cages (maximum of three rats per cage) in a room with controlled temperature (22.3 ± 0.6 °C) and humidity (55.7 ± 2.4% relative humidity), and a 12-h light/dark cycle. The rats were fed a rodent diet (Harlan Teklad, Madison, Wisconsin, USA) and filtered water ad libitum. There were four groups consist of 10 animals (five females and five males) at each dose level: vehicle control (distilled water), low-dose (500 mg/kg), middle-dose (1,000 mg/kg), and high-dose (2,000 mg/kg). When the rats were 8-weeks-of-age, they were exposed to nanocalcium by gavage at a dose of 20 ml/kg. For 14 days, clinical signs and mortalities of all animals were observed once a day and the body weight were measured four times (before administration and on days 1, 7, and 14 after administration). On day 14, all surviving animals were anesthetized with CO2 gas and killed by exsanguination from the aorta. Complete post-mortem examinations were performed on all vital organs.

Repeated-dose 14-day oral toxicity test

This test was performed in accordance with KFDA Notification No. 2012-86 (Toxicity Test Standards of Medicine and Medical Supplies, Revised August 24, 2012) and OECD Test Guidelines 407 (OECD, 1998). The test substance, nanocalcium, was supplied by KFDA and was dispersed in distilled water. Five-week-old male and female, SPF SD rats supplied from ORIENT BIO were acclimated for 7 days before administration. During the acclimation and experimental periods, the rats were housed in stainless wire mesh cages (maximum of three rats per cage) in a room with controlled temperature (21.6 ± 1.2 ^{o}C) and humidity (56.6 ± 8.5% relative humidity) and a 12-h light/dark cycle. The rats were fed a rodent diet (Harlan Teklad) and filtered water ad libitum. There were four groups (n=10 per group; five females and five males) at each dose level: vehicle control (distilled water), low-dose (500 mg/kg), middle-dose (1,000 mg/kg), and high-dose (2,000 mg/kg). When the rats were 6-weeks-of-age they were exposed to nanocalcium by gavage. The total dosing volume was 20 ml/kg but it was administrated twice a day by dividing the total dosing into two 10 ml/kg in 1-2 h intervals. For 14 days, clinical signs and mortalities of all animals were observed twice a day just after administration. The body weight and food consumption were measured once a week. One week after the administration, the eye examination and urinalysis were performed using a CliniTek 50 (SIEMENS, Berlin, Germany). The urinalysis included the following inspection items: glucose, urobilinogen, bilirubin, nitrite, ketone body, leukocyte, specific gravity, protein, occult blood, and pH. At the end of the study, rats fasted one night before the necropsy were anesthetized with CO_2 gas and a blood sample was collected from the abdominal aorta

just prior to killing each animal. They were analyzed for hematology using an ADVIA 2120 apparatus (SIEMENS, Berlin Germany) and a model 7180 clinical biochemistry instrument (Hitachi, Hitachi, Japan). Inspection items in hematology were white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) Mean corpuscular hemoglobin conc. (MCHC), Red cell distribution width (RDW), Platelet (PLT), Mean Platelet Volume (MPV), differential count, Large unstained cells (LUC), and reticulocytes. Inspection items in clinical biochemistry were aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), Gamma(γ)-glutamyl transferase (GGT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine, glucose, total cholesterol, total protein, creatine phosphokinase, Albumin (ALB), total bilirubin, Albumin / Globulin (A/G) ratio, triglyceride, uric acid, calcium (Ca), Inorganic phosphorus (IP), chloride (Cl), magnesium (Mg), sodium (Na), and potassium (K). Each rat was terminated by exsanguination and subjected to a full necropsy with complete post-mortem examinations. The extracted organs were weighed using an electronic balance. Finally, the histopathological examination was conducted for abnormal organs.

Statistical analyses

The differences among the vehicle control and the dosing groups were examined using SPSS for Windows version 12.0 software (SPSS, Chicago, IL, USA). Continuous

data (body weights, food consumption, hematology, blood biochemistry, and organ weights) were analyzed to analyzed supposing that the distribution is normal. Differences among the groups were examined and equal variance was ascertained using the standard one-way analysis of variance (ANOVA). If these tests showed statistical significance, the data were analyzed using the multiple comparison procedure. While, non-continuous data (urinalysis) were converted by scale conversion and analyzed by Chi-squared analysis. A p-value < 0.05 indicated statistical significance.

RESULTS

Characterization of nano- and microcalcium

The size of nano- and microcalcium were determined using ed EF-TEM and FE-SEM. Representative EF-TEM images of nanocalcium and microcalcium are shown in Figures 1A and 1B, respectively. While the average particle sizes could not be determined, nanocalcium particle diameter ranged from 50 nm to 1 μm. The diameter of microcalcium ranged from 1 μm to 10 μm. EF-TEM also revealed variously sized agglomerations of nano- and microcalcium particles. FE-SEM was carried out to observe the surface shape of agglomerated and non-agglomerated calcium. Nanocalcium particles over 100 nm in diameter featured a crystalline agglomerate core (Figure 2A-1), while smaller diameter particles had a non-crystalline portion bonded on the core calcium (Figure 2A-2). Nanocalcium particles developed for use as dietary supplements were agglomerated, so determination of their mean size with EF-TEM and FE-SEM was not feasible. DLS also was not useful in size determination (Figure 3). Nanocalcium particles featured two size groups, one from 200–500 nm and the other exceeding 500 nm. Microcalcium particles ranged from 1.5–30 μm, with many subgroups evident. XRD analysis revealed common aspects of component properties between nano- and microcalcium. Both particle types were single-phased calcium carbonate ($CaCO_3$) with a calcite structure (Figure 4), despite the differing surface morphology and inability to determine average size. Moreover, the specific surface area could not be reliably determined for agglomerated samples. For agglomerates, the

expected number of particles with a specific surface area was disparate; the increase of surface area was smaller than expected for agglomerated particles due to the decrease of their size in the same mass. For safety determinations of nanomaterials, including nanocalcium, it is important to provide information related with size and concentration. To overcome these limitations in the agglomerated samples, the BET method was tried. The specific surface area of nanocalcium and microcalcium was 12.90 ± 0.27 m^2/g and 1.12 ± 0.19 m^2/g, respectively. The surface area of nanocalcium particles exceeded that of microcalcium by 12 times (Table 1).

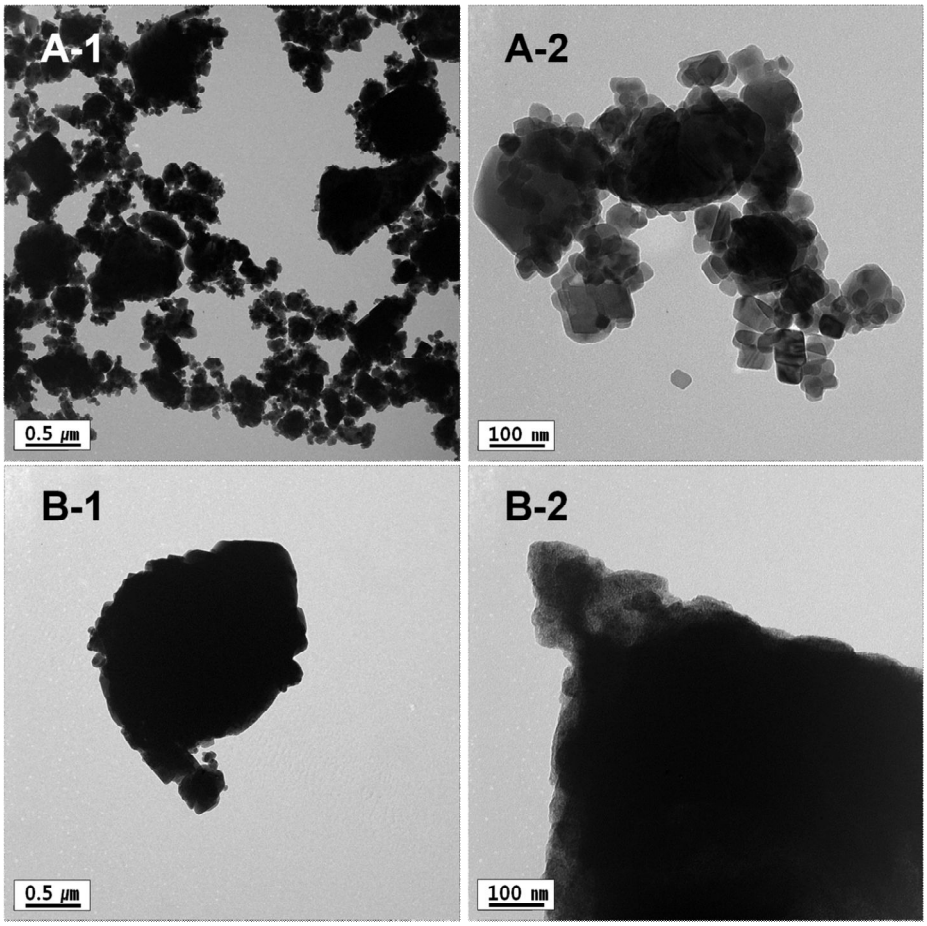

Figure 1 | Energy-filtering transmission electron microscopy images of nanocalcium (A) and microcalcium (B). Nano- and microcalcium particles were polygonal in shape. Particle size varied widely. The diameter of nanocalcium particles ranged from 50 nm to 1 μm, and that of microcalcium particles ranged from 1 μm to 10 μm.

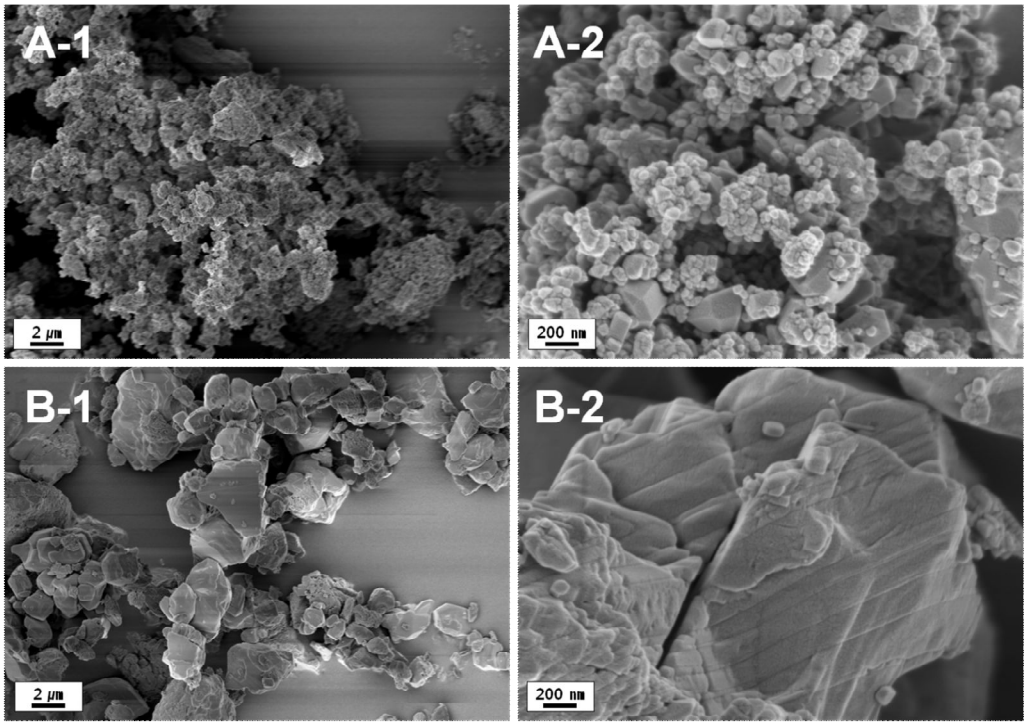

Figure 2 | Field-Emission scanning electron microscopy images of nanocalcium (A) and microcalcium (B) particles. The diameter of nanocalcium particles ranged from 30–200 nm, and that of microcalcium particles ranged from 10–50 μm.

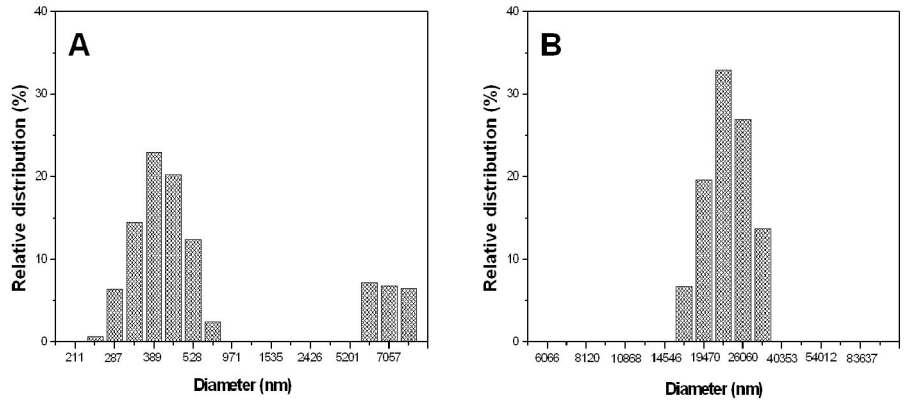

Figure 3 | Dynamic light scattering analysis of (A) nanocalcium and (B) microcalcium particles. The left, middle, and right panel displays intensity, volume, and number in each sample, respectively.

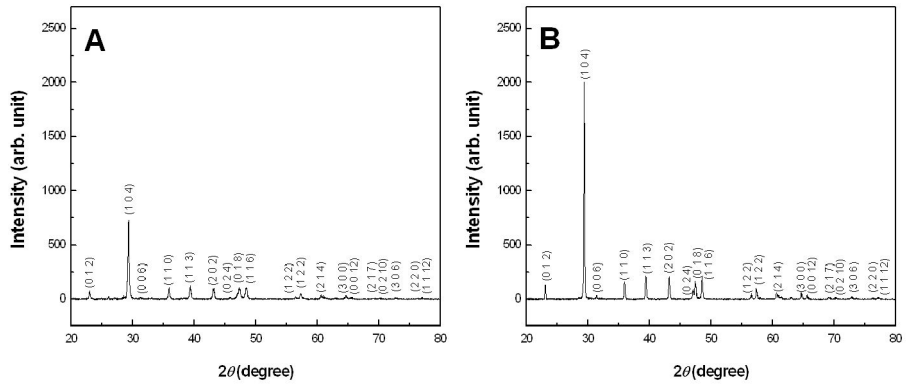

Figure 4 | X-ray diffractometry patterns of nanocalcium (A) and microcalcium (B) particles.

Table 1. Physicochemical analyses of nanocalcium and microcalcium.

	Measuring method (Characteristic)	Results	Note
Nanocalcium	*EF-TEM*	Impossible	Sample agglomerated and ranged from 50 nm to 1μm.
	FE-SEM	Impossible	Various size distributions and agglomerated
	XRD	Calcium carbonate	
	DLS	Two size section (200 nm ~500 nm and > 500 nm)	The majority were 200–500 nm
	BET	12.903 ± 0.2715 m^2/g	(Mean ± SD)
	ESR	Not detected	No ROS signal on their surface.
Microcalcium	*EF-TEM*	Impossible	Samples agglomerated and ranged from 1–50 μm.
	FE-SEM	Impossible	Various size distributions and agglomerated
	XRD	Calcium carbonate	
	DLS	Roughly 20 μm	Samples agglomerated and ranged from 15–40 μm
	BET	1.1221 ± 0.1938 m^2/g	(Mean ± SD)
	ESR	Not detected	No ROS signal on their surface.

ESR spectroscopy and spin-trapping measurements

To detect reactive oxygen species (ROS) from the surface of nanocalcium, ESR signals corresponding to the physicochemical characteristics were analyzed. Hydroxyl radical signal measured using 50 mM DMPO was not detected. Singlet oxygen measured using 4.7 mM TEMP displayed a unique 1:1:1 pattern, but was not appreciably different from the distilled water control. As shown in Figure 5, no ESR signals were evident in nano- and microcalcium samples, indicating the lack of ROS on the surface of both particle types.

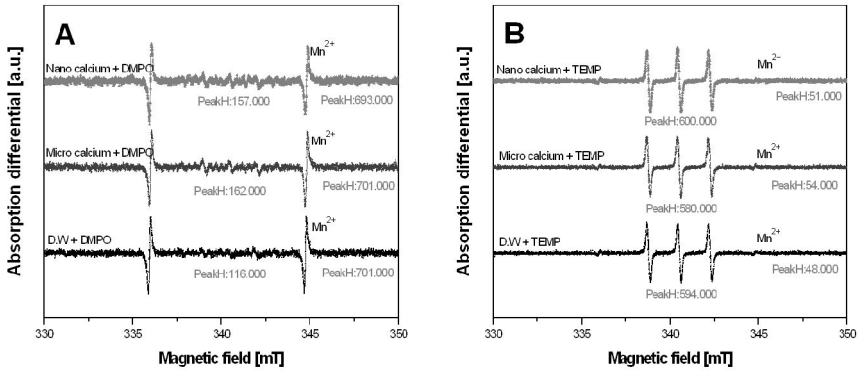

Figure 5 | Electron spin resonance analysis of nanocalcium (upper line), microcalcium (middle line), and control (lower line) in detection of hydroxyl radial (A) and singlet oxygen (B). Hydroxyl radial was analyzed using a spin-trap agent (50 mM DMPO). Singlet oxygen was analyzed using another spin-trap agent (4.7 mM TEMP).

Ca^{2+} release from nano- and microcalcium particles

Ca^{2+} release from nano- and microcalcium particles was assessed using ICP-OES. Table 2 summarizes the concentrations of Ca^{2+} detected at the various pHs. At pH 2, there was no critical difference in the release of Ca^{2+}. However, at pH 5 and pH 7, the Ca^{2+} released from nanocalcium exceeded that from microcalcium by 2–3 times.

Table 2. Concentration of Ca^{2+} released from nano- and microcalcium particles in various solvent for 6 h.

Sample	Solvent	Test concentration of $CaCO_3$ (mg/ml)	Release of Ca^{2+}(µg/ml, n = 3)
			Concentration ± S.D.
Solvent		0	0.058 ± 0.006
Nanocalcium	pH 2	10	593.333 ± 22.038
Microcalcium		10	564.533 ± 26.530
Solvent		0	0.024 ± 0.003
Nanocalcium	pH 5	10	16.747 ± 0.081
Microcalcium		10	7.344 ± 0.077
Solvent		0	0.000 ± 0.000
Nanocalcium	pH 7	10	15.860 ± 0.105
Microcalcium		10	5.560 ± 0.077

Ca^{2+} content of $CaCO_3$ materials was determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

Genotoxicity of nanocalcium

In the chromosome aberration test, nanocalcium did not produce a statistical increase in the number of cells, when compared to conditions with or without metabolic activation. Furthermore, in the presence and absence of the S9 mix, nanocalcium did not induce a statistically significant increase in the number of cells with polyploidy or endoreduplication when compared with the negative control group (Table 3). In an in vivo micronucleus assay, nanocalcium did not induce a significant increase in MNPCEs at 500 mg/kg, 1000 mg/kg, and 2000 mg/kg, as compared to the negative control group (Table 4). Furthermore, there was no significant decrease in the PCE / (PCE+NCE) ratio, which is an indicator of cytotoxicity. No abnormalities were evident at any concentration of administered nanocalcium. Body weight did not differ significantly between treated and negative control groups (data not shown). As expected, MNPCEs in the positive control group were significantly increased compared to the negative control ($P<0.01$), confirming the validity of the experiment. In the bacterial reverse mutation assay, growth inhibition was not observed in any dose level and strain regardless of metabolic activation (Table 5). There was no significant increase in the number of revertant colonies when compared to the negative control in all bacterial strains both with and without metabolic activation. These results support the view that nanocalcium does not produce severe genotoxicity under the experimental conditions.

Table 3. Chromosome aberrations test of nanocalcium in Chinese hamster ovary fibroblasts in the absence or presence of S9 mix.

Exposure[a]	S9 mix	Dose (μg/ml)	No. of total chromosome aberrations (mean)		No. of cells with chromosome aberrations (mean)		PP+ER (Mean)
			(-)Gap	(+)Gap	(-)Gap	(+)Gap	
24-0	-	Negative control	0.5	0.5	0.5	0.5	0
	-	39.06	0.5	1.5	0.5	1.5	0
	-	78.13	0.5	1	0.5	1	0
	-	156.25	1	1.5	1	1.5	0
	-	MMC (0.04)	26.5	26.5	26 *	26	0
6-18	-	Negative control	0	0	0	0	0
	-	39.06	1	1.5	1	1.5	0
	-	78.13	0.5	0.5	0.5	0.5	0
	-	156.25	0.5	1	0.5	1	0
	-	MMC (0.04)	24	24	24 *	24	0
6-18	+	Negative control	0.5	0.5	0.5	0.5	0
	+	78.13	0.5	0.5	0.5	0.5	0
	+	156.25	1	1	1	1	0
	+	312.5	1.5	1.5	1.5	1.5	0
	+	CPA (10)	26.5	26.5	26.5 *	26.5	0

* Significantly different from the negative control at $P<0.05$
[a] Treatment time-recovery time
MMC: Mitomycin C (0.04 μg/ml)
PP: Polyploidy
ER: Endoreduplication
CPA: Cyclophosphamide · H_2O (10 μg/ml)

Table 4. Bone marrow micronucleus test of nanocalcium in ICR mice.

Groups	Dose (mg/kg)	MNPCE/2000 PCEs (Mean±SD, %)	PCE/(PCE+NCE) (Mean±SD)
Vehicle control	0	0.23 ± 0.15	0.42 ± 0.08
nanocalcium	500	0.15 ± 0.11	0.49 ± 0.11
	1000	0.29 ± 0.10	0.52 ± 0.06
	2000	0.21 ± 0.14	0.56 ± 0.04 *
MMC	2.0	9.02 ± 0.66 **	0.37 ± 0.03

Vehicle: distilled water
* Significantly different from the control at $P<0.05$ (One-way ANOVA)
** Significantly different from the control at $P<0.01$ (One-way ANOVA)
MNPCE: PCE with one or more micronuclei
PCE: Polychromatic erythrocyte
NCE: Normochromatic erythrocyte
MMC: Mitomycin C

Table 5. Bacterial reverse mutation test of nanocalcium in the absence or presence of S9 mix.

Metabolic activation	Dose (μg/plate)	Number of colony / plate (Mean ± SD)				
		TA98	TA100	TA1535	TA1537	WP2uvrA
S9 mix (-)	0	20±1.2	95±8.3	7±1.7	4±2.0	54±3.1
	313	24±2.6	74±4.6	6±0.6	3±0.6	50±7.6
	625	18±2.5	79±0.6	7±3.2	4±1.5	47±4.0
	1250	18±1.5	70±1.5	4±0.0	6±1.5	51±6.8
	2500	20±3.1	63±1.5	5±1.5	5±1.5	46±4.4
	5000 †	19±1.0	64±3.2	7±1.0	5±1.0	34±4.0
	Positive	549±78.5[a]	760±104.6[b]	265±62.0[c]	2207±197.4[d]	245±37.8[b]
S9 mix (+)	0	23±2.5	94±2.6	7±2.0	8±1.2	56±6.2
	313	24±5.0	103±12.1	10±2.1	10±1.5	49±1.0
	625	21±3.6	108±3.1	8±2.0	8±2.5	54±1.0
	1250	23±4.4	94±5.9	9±2.9	12±3.2	47±8.7
	2500	23±1.5	93±3.5	7±2.6	11±2.1	53±9.9
	5000	22±1.5	100±11.8	7±2.6	10±1.5	43±10.6
	Positive	351±32.1[e]	995±48.9[f]	183±15.0[g]	252±58.9[h]	344±34.9[g]

[a] Positive control was 0.1 μg/plate of 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
[b] Positive control was 0.01 μg/plate of 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
[c] Positive control was 0.5 μg/plate of Sodium azide
[d] Positive control was 80 μg/plate of 9-Aminoacridine hydrochloride hydrate
[e] Positive control was 0.5 μg/plate of 2-Aminoanthracene
[f] Positive control was 1 μg/plate of 2-Aminoanthracene
[g] Positive control was 2 μg/plate of 2-Aminoanthracene (Continue-next page)

[h] Positive control was 10 μg/plate of 2-Aminoanthracene

†: test substance precipitation

Single-dose acute oral toxicity

Lethal dose 50 (LD_{50}) and oral toxicity of nanocalcium were determined in Sprague-Dawley rats at doses of 500 mg/kg (low-dose), 1,000 mg/kg (middle-dose) and 2,000 mg/kg (high-dose), and compared with vehicle control group. Mortality, clinical signs, body weight, and necropsy findings were determined. Mortalities and unusual clinical signs were not observed and there were normal body weight gains in all animals (data not shown). Necropsy revealed no abnormal gross findings. The collective data indicate that under the conditions of this study, the single-dose acute oral LD_{50} of nanocalcium can exceed 2,000 mg/kg body weight.

Repeated-dose 14-day oral toxicity test

This study was performed to evaluate the 14-day oral repeated dose toxicity and determine the dose levels for 13-week oral repeated dose toxicity of nanocalcium in Sprague-Dawley rats using doses of 0 (vehicle control), 500 (low dose), 1,000 (middle dose), and 2,000 (high dose) mg/kg. Mortality, clinical signs, body weight change, food consumption, eye examination, urinalysis, hematology, blood biochemistry, necropsy, organ weights, and histopathological findings were examined. No deaths or unusual clinical signs were evident. There were no significant differences among groups in most of the tested parameters, such as body weights (Figure 6), food consumptions

(data not shown), urinalysis (data not shown), hematology (Table 6), blood chemistry (Table 7), and organ weights (Table 8). Especially, there was no critical difference in Ca2+ level in the nanocalcium treated rats. Significant differences were observed in food consumption and some aspects of hematology (percentage of neutrophils and percentage of lymphocytes), but they were considered to be unrelated with the applied test substance. Necropsy findings included focal nodule of spleen, small left testis, and adrenal in male rats receiving the dose of 2,000 mg/kg. They were considered as focal increased cellularity of white pulp at kidney, unilateral atrophy/immature at testis, and unilateral aplasia at adrenal in histopathological examination. However, these changes were likely unrelated with the test substance and were considered to be incidental or to have occurred congenitally. Under the conditions of this study, there were no toxicological significances related to the test substance. OECD Test Guideline 408 (OECD, 1998) suggests 1,000 mg/kg as the limited dose level on repeated dose toxicity test. On the basis of the present results, 1,000 mg/kg is recommended as the high dose level for repeated dose 13-week oral toxicity study of nanocalcium.

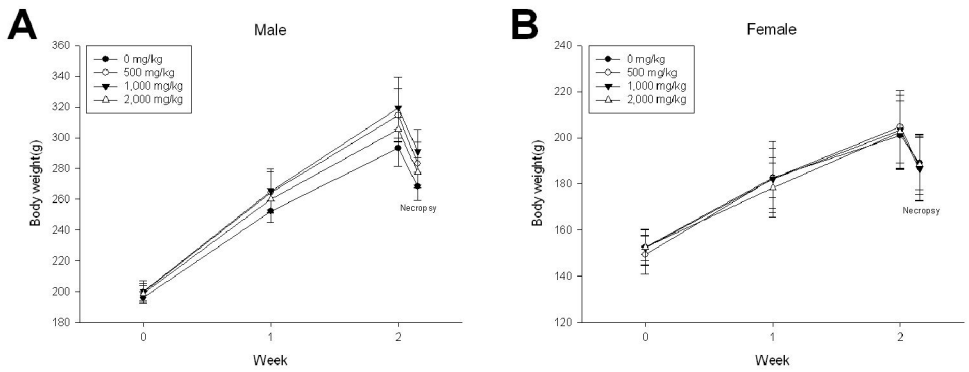

Figure 6 | Body weight changes of male (A) and female (B) rats orally treated with nanocalcium for 14 days. Body weight was measured 1st week and 2nd week. Each point represents the mean ± S.D. No statistically significant changes were observed.

Table 6. Hematology in rats treated orally with nanocalcium for 14 days.

TEST ITEM (UNIT)	Dose (mg/kg)			
	0	500	1,000	2,000
MALE	**Mean ± S.D.**			
WBC[a] (K/μl)	7.00 ±0.63	8.02 ±1.35	10.06 ±1.84	8.34 ±3.66
NE[b] (K/μl)	0.84 ±0.18	1.25 ±0.46	0.97 ±0.18	1.32 ±0.56
LY[c] (K/μl)	5.95 ±0.70	6.56 ±1.31	8.84 ±1.61	6.75 ±2.97
MO[d] (K/μl)	0.12 ±0.03	0.13 ±0.03	0.13 ±0.05	0.15 ±0.08
EO[e] (K/μl)	0.04 ±0.02	0.03 ±0.01	0.04 ±0.01	0.05 ±0.03
BA[f] (K/μl)	0.00 ±0.01	0.01 ±0.01	0.01 ±0.00	0.01 ±0.01
NEP[g] (%)	12.1 ±3.3	15.8 ±5.6	9.6* ±0.8	15.9 ±1.4
LYP[h] (%)	84.8 ±3.7	81.7 ±5.8	87.9* ±0.5	81.1 ±1.7
MOP[i] (%)	1.7 ±0.5	1.6 ±0.3	1.3 ±0.4	1.8 ±0.5
EOP[j] (%)	0.6 ±0.2	0.4 ±0.2	0.3 ±0.1	0.6 ±0.2
BAP[k] (%)	0.1 ±0.1	0.1 ±0.1	0.1 ±0.0	0.1 ±0.0
RBC[l] (M/μl)	7.18 ±0.37	7.17 ±0.27	6.93 ±0.29	7.03 ±0.34
Hb[m] (g/dl)	15.0 ±0.7	14.7 ±0.5	14.4 ±0.4	14.4 ±0.8
HCT[n] (%)	47.1 ±2.5	46.6 ±0.5	45.8 ±1.3	45.2 ±2.9
MCV[o] (fl)	65.5 ±1.2	65.1 ±3.0	66.0 ±2.9	64.3 ±1.8
MCH[p] (pg)	20.9 ±0.4	20.6 ±0.6	20.8 ±0.9	20.5 ±0.6
MCHC[q](g/dl)	31.9 ±0.5	31.6 ±1.1	31.5 ±0.4	31.8 ±0.9
RDW[r] (%)	11.6 ±0.3	11.9 ±0.6	11.9 ±0.2	11.7 ±0.4
PLT[s] (K/μl)	1367 ±128	1457 ±164	1363 ±138	1337 ±176
MPV[t] (fl)	9.1 ±0.3	9.1 ±0.2	9.1 ±0.2	9.1 ±0.3
LUC[u] (%)	0.06 ±0.02	0.05 ±0.03	0.07 ±0.04	0.06 ±0.04
Reti[v] (%)	4.4±0.4	5.1±1.5	4.5±0.2	4.2±0.4
FEMALE	**Mean ± S.D.**			
WBC[a] (K/μl)	6.38±2.04	8.67±2.46	7.54±4.01	5.81±1.28
NE[b] (K/μl)	0.63±0.26	0.67±0.16	0.64±0.38	0.50±0.20
LY[c] (K/μl)	5.53±1.80	7.71±2.28	6.67±3.64	5.10±1.13
MO[d] (K/μl)	0.10±0.02	0.12±0.06	0.14±0.09	0.11±0.02
EO[e] (K/μl)	0.05±0.02	0.06±0.02	0.04±0.01	0.03±0.02
BA[f] (K/μl)	0.00±0.01	0.00±0.01	0.00±0.00	0.01±0.01
NEP[g] (%)	10.0±2.7	8.2 ±2.5	8.5±2.3	8.6±2.7
LYP[h] (%)	86.6±2.4	88.5±2.4	88.4±3.2	87.8±2.9
MOP[i] (%)	1.6±0.3	1.4±0.5	1.8±0.7	2.0±0.3

EOP[j] (%)	0.7 ±0.4	0.7±0.2	0.5±0.2	0.4±0.3
BAP[k] (%)	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
RBC[l] (M/μl)	7.21±0.42	6.93±0.45	6.92±0.20	7.20±0.48
Hb[m] (g/dl)	14.5±0.5	14.3±0.5	14.4±0.6	14.9±1.1
HCT[n] (%)	44.2±2.1	42.4±2.1	43.3±2.5	45.3±3.7
MCV[o] (fl)	61.3±2.5	61.3±2.1	62.5±2.1	62.9±2.5
MCH[p] (pg)	20.1±0.8	20.7±1.0	20.9 ±0.5	20.7±0.8
MCHC[q] (g/dl)	32.9±0.5	33.8±0.7	33.4±0.6	33.0±0.7
RDW[r] (%)	10.8±0.1	11.1±0.5	10.7±0.3	11.1±0.4
PLT[s] (K/μl)	1459±189	1359±248	1608±332	1472±194
MPV[t] (fl)	8.9±0.3	8.7±0.5	9.0±0.8	9.2±0.7
LUC[u] (%)	0.07±0.04	0.10±0.05	0.06±0.03	0.06±0.02
Reti[v] (%)	2.8±0.5	3.0±0.2	2.7±0.5	2.7±0.7

Each group consisted of five animals of both sexes. Values are mean±standard deviation. *Significant difference compared with 2,000 mg/kgvalue, $P<0.05$. *a*: White blood cell, *b*: Neutrophils, *c*: Lymphocyte, *d*: Monocyte, *e*: Eosinophil, *f*: Basophil, *g*: Percent of neutrophils, *h*: Percent of lymphocyte, *i*: Percent of monocyte, *j*: Percent of eosinophil, *k*: Percent of basophil, *l*: Red blood cell, *m*: Hemoglobin, *n*: Hematocrit, *o*: Mean corpuscular volume, *p*: Mean corpuscular hemoglobin, *q*: Mean corpuscular hemoglobin concentration, *r*: Red cell distribution width, *s*: Platelet, *t*: Mean platelet volume, *u*: Large unstained cell, *v*: Reticulocyte

Table 7. Blood chemistry in rats treated per orally with nanocalcium for 14 days.

TEST ITEM (UNIT)	Dose (mg/kg)			
	0	500	1,000	2,000
MALE	**Mean ± S.D.**			
AST[a] (IU/l)	157 ±21	144 ±19	165 ±29	149 ±25
ALT[b] (IU/l)	32 ±2	34 ±4	43 ±11	35 ±5
ALP[c] (IU/l)	941 ±231	840 ±136	876 ±125	797 ±213
GGT[d] (IU/l)	0.0 ±0.0	0.0 ±0.0	0.2 ±0.4	0.2 ±0.4
LDH[e] (IU/l)	1811 ±492	1447 ±479	1716 ±703	1550 ±452
BUN[f] (mg/dl)	14.0 ±1.8	14.0 ±0.6	13.5 ±1.3	13.9 ±1.6
CRE[g] (mg/dl)	0.42±0.06	0.42±0.03	0.40±0.03	0.41±0.03
GLU[h] (mg/dl)	130±14	150 ±50	141 ±33	144±24
CHO[i] (mg/dl)	84±10	70 ±16	88 ±22	70 ±21
TP[j] (g/dl)	5.6 ±0.2	5.6 ±0.1	5.4 ±0.2	5.5 ±0.2
CPK[k] (U/l)	1114 ±306	969 ±228	1125 ±422	1086 ±371
ALB[l] (g/dl)	2.4 ±0.1	2.4 ±0.0	2.3 ±0.0	2.4 ±0.1
TBIL[m] (mg/dl)	0.04±0.01	0.03±0.01	0.02±0.01	0.02±0.02
A/G ratio[n]	0.74±0.03	0.74±0.04	0.72±0.06	0.76±0.03
TG[o] (mg/dl)	42 ±12	45 ±16	51 ±16	47 ±12
UA[p] (mg/dl)	2.8 ±1.1	2.4 ±0.9	2.5 ±0.4	2.5 ±0.5
Ca[q] (mg/dl)	10.7 ±0.2	10.4 ±0.7	10.5 ±0.2	10.3 ±0.5
IP[r] (mg/dl)	11.1 ±0.8	10.8 ±0.7	10.9 ±0.8	11.2 ±1.2
Cl[s] (mmol/l)	106 ±1	105 ±2	106 ±2	106 ±2
Mg[t] (mg/dl)	2.8 ±0.6	2.7 ±0.3	2.6 ±0.1	2.5 ±0.1
Na[u] (mmol/l)	143 ±1	142 ±1	143 ±1	143 ±1
K[v] (mmol/l)	5.9 ±0.1	5.3 ±0.5	5.7 ±0.2	5.7 ±0.6
FEMALE	**Mean ± S.D.**			
AST[a] (IU/l)	135±24	145±39	150±40	136±48
ALT[b] (IU/l)	26±4	26±6	26±3	26±7
ALP[c] (IU/l)	587±197	498±88	513±115	504±131
GGT[d] (IU/l)	0.2±0.4	0.8±0.4	0.2±0.4	0.2±0.4
LDH[e] (IU/l)	1343±525	1540±893	1545±622	1326±834

BUN[f] (mg/dl)	15.0±1.4	16.0±2.3	14.1±2.7	14.2±2.8
CRE[g] (mg/dl)	0.40±0.05	0.42±0.05	0.45±0.06	0.41±0.04
GLU[h] (mg/dl)	101±10	113±7	107±23	100±11
CHO[i] (mg/dl)	77±10	88±10	72±14	91±24
TP[j] (g/dl)	5.9±0.5	5.7±0.2	5.7±0.2	5.7±0.3
CPK[k] (U/l)	1109±473	1011±527	1064±456	989±536
ALB[l] (g/dl)	2.6±0.3	2.5±0.1	2.5±0.1	2.5±0.1
T-BIL[m] (mg/dl)	0.03±0.01	0.04±0.01	0.03±0.02	0.03±0.01
A/G ratio[n]	0.82±0.05	0.80±0.06	0.78±0.04	0.80±0.02
TG[o] (mg/dl)	28±11	30±13	30±13	48±29
UA[p] (mg/dl)	2.2±0.6	2.3±0.3	2.4±0.4	2.3±0.5
Ca[q] (mg/dl)	10.6±0.1	10.5±0.3	10.3±0.5	10.7±0.6
IP[r] (mg/dl)	10.5±0.5	11.0±0.5	11.0±0.9	11.2±0.2
Cl[s] (mmol/l)	106±2	106±2	106±1	106±2
Mg[t] (mg/dl)	2.9±0.3	2.8±0.2	2.9±0.3	2.9±0.3
Na[u] (mmol/l)	142±1	141±1	141±2	142±1
K[v] (mmol/l)	5.9±0.7	6.1±1.2	6.1±0.8	6.2±0.8

Each group consisted of five animals of both sexes. Value are the mean±standard deviation. *a*: Aspartate aminotransferase, *b*: Alanine aminotransferase, *c*: alkaline phosphatase, *d*: Gamma(γ)-glutamyl transferase, *e*: Lactate dehydrogenase, *f*: Blood urea nitrogen, *g*: Creatinine, *h*: Glucose, *i*: Total cholesterol, *j*: Total protein, *k*: Creatine phosphokinase, *l*: Albumin, *m*: Total bilirubin, *n*: Albumin/Globulin ratio, *o*: Triglyceride, *p*: Uric acid, *q*: Calcium, *r*: Inorganic phosphorus, *s*: Chloride, *t*: Magnesium, *u*: Sodium, *v*: Potassium

Table 8. Organ weights in rats treated per orally with nanocalcium for 14 days.

ORGAN	Dose (mg/kg)			
	0	500	1,000	2,000
Male	**Mean ± S.D.**			
Body weight	268.45±9.06	283.13±14.01	291.02±14.26	277.45±9.88
Testis (Lt.)	1.4341±0.1257	1.4051±0.0429	1.3660±0.1956	1.2341±0.4618
Testis (Rt.)	1.4026±0.1349	1.4322±0.0614	1.3537±0.1851	1.4678±0.1403
Prostate	0.4041±0.0842	0.4043±0.1142	0.3869±0.0727	0.3696±0.0682
Spleen	0.6005±0.1105	0.7994±0.2393	0.7258±0.0654	0.7457±0.1503
Liver	8.4166±0.4019	9.3058±0.9886	9.4535±1.0318	8.5626±0.4509
Adrenal gland (Lt.)	0.0281±0.0041	0.0316±0.0040	0.0296±0.0035	0.0239±0.0086
Adrenal gland (Rt.)	0.0259±0.0031	0.0286±0.0029	0.0273 ±0.0031	0.0268±0.0030
Kidney (Lt.)	1.0696±0.1033	1.2041±0.0547	1.1669±0.2054	1.1334±0.1108
Kidney (Rt.)	1.1273±0.1389	1.2247±0.0895	1.1636±0.1388	1.1732±0.0647
Heart	1.0062±0.0939	1.0637±0.0556	1.0934±0.0702	1.0766±0.0396
Lung	1.2356±0.1005	1.4054±0.1464	1.3416±0.1532	1.4252±0.2591
Brain	1.8860±0.0873	1.9088±0.0324	1.9718±0.0328	1.9123±0.1035
Thymus	0.5885±0.1583	0.6018±0.1580	0.7551±0.0920	0.5430±0.0643
Female	**Mean ± S.D.**			
Body weight	189.17±11.56	187.29±14.35	186.44±13.82	188.34±13.01
Ovary (Lt.)	0.0434±0.0056	0.0380±0.0126	0.0464±0.0107	0.0370±0.0044
Ovary (Rt.)	0.0403±0.0055	0.0382±0.0074	0.0469±0.0068	0.0372±0.0107
Uterus	0.4398±0.2482	0.3614±0.0636	0.4817±0.1364	0.5619±0.2637
Spleen	0.4527±0.0615	0.4689±0.0752	0.4817±0.1200	0.4563±0.0519
Liver	6.5668±0.5396	6.2212±0.7046	6.4795±0.5037	6.1319±0.7056
Adrenal gland (Lt.)	0.0318±0.0053	0.0302±0.0047	0.0355±0.0037	0.0321±0.0042
Adrenal gland (Rt.)	0.0312±0.0056	0.0279±0.0035	0.0335±0.0038	0.0296±0.0017
Kidney (Lt.)	0.8609±0.1147	0.8170±0.1091	0.8894±0.1395	0.8126±0.1197
Kidney (Rt.)	0.8760±0.1122	0.8238±0.1069	0.8738±0.1320	0.8318±0.1208
Heart	0.7755±0.0756	0.7378±0.1023	0.7614±0.0392	0.7664±0.0644
Lung	1.1800±0.1608	1.0658±0.1025	1.2599±0.1781	1.3749±0.4174
Brain	1.8890±0.0822	1.8071±0.0900	1.8545±0.0720	1.8454±0.0624
Thymus	0.5230±0.0562	0.6377±0.1538	0.5371±0.1956	0.5706±0.1815

DISCUSSION

Nanotechnology is rapidly growing and will undoubtedly have both beneficial and toxicological effect and consequences on human beings and the environment (Arora et al., 2012). Nanotechnology promises to far exceed the impact of the Industrial Revolution and is projected to become a US$ 1 trillion market by 2015 (Drobne, 2007). The importance of nanotechnology to our lives is beyond debate. Yet, potential adverse effects need to be studied. Nanotoxicology was proposed as a new branch of toxicology to address the gaps in knowledge and to specifically address the adverse health effects likely to be caused by nanomaterials (Donaldson et al., 2004). In various fields, including those involving food, many nanomaterials and nanotechnology are already on the market. Currently, there are no special regulations for food-related nanotechnology. The Food and Drug Administration (FDA) regulates on a product-by-product basis and points out many products made using currently regulated nanoparticles (FDA, 2004; Weiss et al., 2006).

Calcium is vitally important for maintaining health. Calcium deficiency leads to osteoporosis. However, the absorption efficiency of calcium is low. To overcome this limitation, the use of nano-sized calcium has been explored. Nanocalcium shares properties with microcalcium. But, there may be differences in ADME, including toxicity, as a consequence of the increased specific surface area of nanoparticles.

The possible toxicity of nanoparticles in food is an important issue, even in the absence of specific legislation mandating such studies. The physicochemical properties

of the nanomaterials including nanocalcium needs to be well characterized and the use of appropriate dose metrics is very important for toxicity testing. The characterization of nanofoods before and after in vitro and in vivo administration is also needed in screening studies. Huang et al. (2009) used TEM analyses to demonstrate that administering nanocalcium carbonate and nanocalcium citrate can enhance the serum calcium concentration and maintain the whole body bone mineral density in ovariectomized mice. Hu et al. (2010) estimated the safety of nanocalcium using an in vitro assay conducted with bone marrow mesenchymal stem cells. In this report, they provided physico-chemical information through TEM, SEM, XRD and FT-IR. Park et al. (2008) found that consumption of nanocalcium enriched milk resulted in increased urinary excretion of calcium and decreased excretion of deoxypyridinoline and hydroxyproline in ovary ectomized rats. Despite the prior knowledge concerning characterization, efficiency, and safety estimation of nanocalcium, more information on the physico-chemical properties and genotoxicity of nanocalcium is needed.

Presently, we analyzed nanocalcium and microcalcium samples by EF-TEM, FE-SEM, DLS and XRD method. EF-TEM revealed nanocalcium and microcalcium diameters ranging from 50 nm to 1μm (Figure 1A) and 1 μm to 10 μm (Figure 1B), respectively. Both samples agglomerated, which prevented accurate determination of their average size by EF-TEM (Figure 1). To better gauge their agglomerated shape, we used SEM (Figure 2). To determine hydrodynamic diameters, both samples were analyzed by DLS, which revealed various size fractions (Figure 4). However, despite the size different, the same crystalline property was evident by XRD (Figure 3). BET-

determined specific surface area of nanocalcium was 12.90 ± 0.27 m^2/g and that of microcalcium was 12-times smaller, at 1.12 ± 0.19 m^2/g (Table 1). Previously, we reported that ROS formation in silver and zinc oxide nanoparticles and linked ROS formation with toxicity (Kim et al., 2007; Park et al., 2011). Presently, ESR did not reveal a ROS signal in either particle type (Table 1).

ICP-OES revealed that the release of 2–3 times more Ca^{2+} from nanocalcium than from microcalcium at pH 5 and pH 7 (Table 2). For nanomaterials like calcium, which can ionize and agglomerate, it may be important to provide information for specific surface area and degree of ionization to asset their toxicity and toxic mechanism.

Genotoxicity was presently explored using a chromosomal aberration test, micro nucleus assay, and bacterial reverse mutation assay in accordance with KFDA notification and OECD Test Guidelines. Nanocalcium did not show any apparent genotoxicity in the recommended high concentration (Tables 3, 4, and 5).

In vivo toxicity testing, also done in accordance with KFDA notification and OECD Test Guideline, involved four groups (10 rats per group, always comprising five females and five males) at each dose level. No severe toxicity was observed in the single-dose acute oral toxicity test and the repeated-dose 14-day oral toxicity test (Figure 6, Tables 6, 7, and 8).

Nanocalcium did not pose genotoxicity and in in vivo toxicity problems in our experimental conditions. In the United States Toxic Substances Control Act (TSCA) inventory, calcium carbonate (CAS# 471-34-1) is classified in the group with the least toxicity (LD_{50} of calcium carbonate, 6450 mg/kg). Presently, nanocalcium exhibited

the same physicochemical properties and was also regarded as having low toxicity in vivo.

Yet, we should be hasty in concluding that toxicity of nanocalcium is same or different as microcalcium. Nanocalcium has a large specific surface area and a greater potential for ionization (Tables 1 and 2). These properties of nanocalcium will lead to different results in long-term monitoring. The present BET and ICP-OES analyses highlight the need for long-term monitoring after administration, due to their differential of ionization derived from their different specific surface areas. Differential toxicity could exist in sub-chronic tests of nanocalcium; thus, it is not enough to provide size information based on TEM, SEM, and DLS in the case of agglomerated samples to explain their differential toxicity. The current findings indicate that BET and ICP-OES may provide more reasonable information about the differential between nano-sized calcium and microcalcium by calculating the surface area of agglomerated nano-materials.

We hope that this report will contribute to the determination of the concentration of the high dose group in 13-week oral repeated dose toxicity test of nanocalcium, and helps indicate the utility of BET in measuring the specific surface area and of ICP-OES in determining the degree of ionization in agglomerated nanomaterials.

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국문 논문 초록

물리화학적 특성에 기초한 나노칼슘의 안전성 평가

(지도 교수: 조 명 행)

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이 연구는 물리 화학적 특성의 기초하여 나노칼슘의 잠재적인 독성을 평가하기 위해 수행되었다. 한국 식품의약품안전청으로부터 제공된 7개의 샘플을 에너지 여과 투과 전자 현미경 검사 (EF-TEM), X선 회절분석 (XRD)를 이용한 성분분석, 그리고 에너지 분산형 분광분석법을 맹검법으로 시험한 후에 나노칼슘과 마이크로칼슘 2개의 샘플을 다음의 시험을 위해 선택하였다. 나노칼슘과 마이크로칼슘의 특성은 EF-TEM, FE-TEM, XRD, 동적 광산란, 그리고 전자스핀 공명법으로 평가되었다. 두가지 시료 모두 응집이 됨이 관찰되었고, 활성산소 역시 생성되지 않았다. N_2 Brunauere Emmette Teller (BET) 방법으로 측정된 나노칼슘과 마이크로칼슘의 비표면적은 각각 12.90 ± 0.27 m2/g, 그리고 1.12 ± 0.19 m^2/g였다.

유도결합플라즈마원자분광기(Inductively coupled plasma optical emission spectrometry)분석으로 pH 5 와 7에서 칼슘이온의 방출이 마이크로칼슘과 비교하여 나노칼슘에서 2~3배 증가됨을 보였다. 나노칼슘의 안전성을 평가하기 위해 유전독성시험과 Sprague-Dawley 쥐를 이용한 단회투여 독성시험 그리고 반복 투여 경구독성시험을 수행하였다. BET와 ICP-OES 분석결과는 나노칼슘의 비표면적이 더 크고 이온화 가능성이 더 크다는 것을 보여준다. 유전독성시험과 in vivo 독성시험에서 나노칼슘의 독성은 보이지 않았다. 하지만 나노칼슘은 그것의 이온화와 비

표면적의 차이 때문에 장기적인 모니터링이 필요하다.

주요어: 나노칼슘, 유전독성, 물리화학적 특성, 비표면적

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