Biocompatibility and bone tissue compatibility of alumina ceramics reinforced with carbon nanotubes

Nobuhide Ogihara[1], Yuki Usui[2], Kaoru Aoki[1], Masayuki Shimizu[1], Nobuyo Narita[1], Kazuo Hara[1], Koichi Nakamura[1], Norio Ishigaki[1], Seiji Takanashi[1], Masanori Okamoto[1], Hiroyuki Kato[1], Hisao Haniu[3], Naoko Ogiwara[4], Noboru Nakayama[5], Seiichi Taruta[5], and Naoto Saito[6,†]

[†] *Author for correspondence*

[1]*Department of Orthopaedic Surgery, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto, 390-8621, Japan*

[2]*Research Center for Exotic Nanocarbons, Asahi 3-1-1, Matsumoto, 390-8621, Japan*

[3]*Institute of Carbon Science and Technology, Shinshu University, Asahi 3-1-1, Matsumoto, 390-8621, Japan*

[4]*Department of Laboratory Medicine, Shinshu University Hospital, Asahi 3-1-1, Matsumoto, 390-8621, Japan*

[5]*Faculty of Engineering, Shinshu University, Wakasato 4-17-1, Nagano, 380-8553, Japan*

[6]*Departments of Applied Physical Therapy, Shinshu University School of Health Sciences, Asahi 3-1-1, Matsumoto, 390-8621, Japan*

ABSTRACT

Aims: The addition of carbon nanotubes (CNTs) remarkably improves the mechanical characteristics of base materials. CNT/alumina ceramic composites are expected to be highly functional biomaterials useful in a variety of medical fields. Biocompatibility and bone tissue compatibility were studied for the application of CNT/alumina composites as biomaterials.

Methods & results: Inflammation reactions in response to the composite were as mild as those of alumina ceramic alone in a subcutaneous implantation study. In bone implantation testing, the composite showed good bone tissue compatibility and connected directly to new bone. An *in vitro* cell attachment test was performed for osteoblasts, chondrocytes, fibroblasts, and smooth muscle cells, and CNT/alumina composite showed cell attachment similar to that of alumina ceramic.

Discussion & conclusion: Owing to proven good biocompatibility and bone tissue compatibility, the application of CNT/alumina composites as biomaterials that contact bone, such as prostheses in arthroplasty and devices for bone repair, are expected.

Keywords: carbon nanotubes, alumina ceramics, composite, biocompatibility, bone tissue, cell attachment, safety

Abbreviations

ALP, alkaline phosphatase; CNT, carbon nanotube; DAPI, 4′, 6-diamino-2-phenylindole; DMSO, dimethyl sulfoxide; HE, hematoxylin and eosin; HIP, hot isostatic pressing; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; SEM, scanning electron microscopy

Introduction

A variety of ceramic products have been clinically applied as biomaterials, and their advantages include high biocompatibility, *in vivo* stability, and superior mechanical characteristics compared to biomaterials such as synthetic polymers, resins, and metals [1-13]. Alumina ceramics are particularly effective as biomaterials owing to their high strength, resistance to wear, and smooth surface [6-8]. They are particularly useful in bone repair in orthopedic surgery, and have composed many products for socket or head of hip joint prostheses for more than 20 years [8]. In addition, these ceramic products have already been used clinically in the femoral components of knee joint prostheses and in ankle joint prostheses [9,10]. In other medical fields, ceramics are used as artificial bone to repair cranial and orbital bone defects and as dental and cochlear implants, among other uses [11-15].

Alumina ceramic products have drawbacks, however. Despite their higher processing accuracy and *in vivo* stability, they have lower strength and greater risk of damage [16-18]. Ideally, these drawbacks could be overcome through the development of new ceramics preserving high accuracy, *in vivo* stability, and high strength. Advances in the development of ceramics for biomaterials have not yet

achieved significant improvements in performance, however [19]. Carbon nanotubes (CNTs) are a novel class of light and strong materials known to impart remarkable improvements in mechanical characteristics when combined with base materials. Thus, research and development of new materials that include CNTs are ongoing in several industrial fields [20,21]. In our study, CNT/alumina composites, novel materials in which CNTs are added as reinforcement for an alumina ceramic base material, were developed to improve the mechanical properties of alumina ceramic while preserving its basic advantages. This combination yielded remarkable improvements in fracture toughness [22].

Biocompatibility and safety—the most important properties for biomaterials—must be confirmed to move forward with clinical application of CNT/alumina composites [23,24]. In addition, compatibility with bone is also important, because ceramics are often components of biomaterials used in bone treatment. Alumina ceramics, which are used as base materials for biomaterials, have been proven to have excellent biocompatibility and bone tissue compatibility through a long history of clinical experience [6,7]. This study is the first to clarify the biocompatibility and bone tissue compatibility of a CNT/alumina composite in comparison with alumina ceramic *in vivo* and *in vitro*.

Materials & methods

Alumina ceramic and CNT/alumina composites

Multi-walled CNTs (VGCF-S, Showa Denko, Tokyo, Japan) with a fiber diameter of approximately 100 nm and fiber length of approximately 10 μm manufactured using a vapor-growth method were used for this study. CNTs were homogenously dispersed in ethanol using an ultra-fine grinding machine similar to a jet mill. During this treatment, CNTs were passed through slit-like channels with ethanol solution under high pressure (200 MPa). Aggregated CNTs underwent strong mechanical stress, including shear and impulsive forces, and were homogenously dispersed in the ethanol. After CNT dispersion, the ethanol solution was mixed with high-purity aluminum powder (Al_2O_3, mean particle diameter 0.1 μm; TM-DAR, Taimei Chemicals, Nagano, Japan) for 24 h through ball milling. The dried powder mixture containing 0.8 wt% CNTs was compacted via cold isostatic pressing at 200 MPa. The resulting green compacts were fired at 1350°C for 0.5 h under vacuum and then treated with hot isostatic pressing at 1350°C and 180 MPa for 0.5 h in a nitrogen atmosphere to yield a CNT/alumina composite.

Alumina ceramic control specimens were fabricated with the same high-purity alumina powder used for the composite. The powder was compacted

with cold isostatic pressing at 200 MPa, fired at 1300°C for 2 h in air, and then treated with hot isostatic pressing at 1300°C and 180 MPa for 2 h in a nitrogen atmosphere to yield alumina ceramic. Different sintering times and temperatures were used for the CNT/alumina composite and the alumina ceramic specimens because the ceramic was prepared under conditions determined by a pilot study to achieve maximum fracture toughness.

Test specimens for subcutaneous implantation and cell attachment studies were prepared by slicing the prepared alumina ceramic and CNT/alumina composite (diameter, ~9 mm; thickness, ~5 mm) to make disks (diameter, ~9-mm; thickness, ~0.8 mm). Both sides of the disks were polished with a diamond polishing plate (125 μm, then 45 μm), and test implants with an approximate 0.5-mm thickness were prepared. The surface roughness and mean grain size of the alumina ceramic and CNT/alumina composite specimens were observed using a by laser microscope (VK-8500, Keyence, Osaka, Japan). Cylindrical alumina ceramic and CNT/alumina composite specimens with diameters of 2 mm and lengths of 6.0 mm were also prepared for the bone implantation study.

Each specimen was tested for mechanical strength as follows. Bending strength was measured with a three-point bending test using a mechanical

testing machine (1123RF55, Instron, MA, USA). Young's modulus, which represents the ratio of strain to tension or compression, was measured using an ultrasonic thickness gage (Panametrics-NDT 35, Olympus, Tokyo, Japan). Vickers hardness was measured using a Vickers hardness meter (VMT-7, Matsuzawa, Akita, Japan). Fracture toughness was measured with an indentation fracture method using a Vickers hardness meter.

Subcutaneous implantation study

Six-week-old male ddY mice underwent surgery after being anesthetized through inhalation of diethyl ether. Specimens of alumina ceramic or CNT/alumina composite with a 9.0-mm diameter and a 0.5-mm thickness were implanted in the dorsal subcutaneous tissue (each group consisted of 3 subjects). Specimens were collected with peripheral skin and muscle tissue after 1 or 4 weeks.

Collected tissue was fixed with 20% neutral buffered formalin solution, and the specimen was extracted without tissue destruction. The tissue was embedded in paraffin, sliced, stained with hematoxylin and eosin (HE), and observed using a light microscope (IX71, Olympus, Tokyo, Japan).

The numbers of lymphocytes, plasma cells, neutrophils, and macrophages in

the tissue in contact with the ceramic specimen were counted in 1 field of the optical microscope (400×).

Bone implantation study

15-week-old male Japanese white rabbits were used for bone implantation study. After general anesthesia was induced using intravenous pentobarbital, a 20-mm section was made in the frontal side of the rabbit thigh and the femur was exposed. Bone defects with diameters of 2.5 mm and depths of 60 mm were prepared using a drill, cylindrical specimens of alumina ceramic or CNT/alumina composite with 2.0-mm diameters and 6.0-mm lengths were embedded in the defect, and the wound was closed. Twelve or 24 weeks after surgery, the rabbits were killed via intravenous anesthesia overdose and their bilateral femurs were collected. The femurs were fixed with 20% neutral buffered formalin solution, embedded in methyl methacrylate (Osteoresin Embedding Kit 297-56001, Wako Pure Chemical Industries, Osaka, Japan), and sliced with a saw microtome (SP1600, Leica Microsystems, Tokyo, Japan). Thin-slice specimens were stained with HE and observed using the light microscope. Each group consisted of 5 subjects.

All animal experimentation procedures were carried out in compliance with

the guidelines of the institutional animal care committee of Shinshu University.

Cell attachment test

Four types of cells were cultured to study cell attachment to the surface of the alumina ceramic or CNT/alumina composite specimens. Osteoblasts isolated and cultured from the calvarium of neonatal rats were used. The other cell types studied were mouse embryonic chondrocytes (ATDC5, Riken BRC Cell Bank, Tsukuba, Japan), mouse embryonic fibroblasts (C3H/HeN-emb, Riken BRC Cell Bank), and human smooth muscle cells (CC-2579, Lonza Walkersville, Walkersville, MD, USA). These cells were cultured with alpha modified Eagle's medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% 200 mM L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). Disks (9.0-mm diameter, 0.5-mm thickness) of alumina ceramic or CNT/alumina composite were placed in a 12-well plate (3 disks per well), and cells were seeded at a density of 3500 cells/cm^2. After culture under general conditions (a humidified, 5% CO_2/95% air environment), the number of cells that attached to the specimens was counted after 1, 3, 6, 24, 72, and 168 h. The number of osteoblasts attached to the standard cell culture plate

was also counted for reference.

Cells that were not attached were washed from the specimen with phosphate buffered saline (PBS), and the attached cells were fixed with formaldehyde. The nuclei were then stained and visualized using 4′, 6-diamino-2-phenylindole (DAPI; VECTASHIELD Mounting Medium with DAPI, Vector, Burlingame, CA, USA). Attached cells were counted in 5 randomly selected fields per specimen under a fluorescence microscope (IX71, Olympus, Tokyo, Japan). All experiments were run in triplicate and repeated 3 times [25].

The morphology and attachment position of osteoblasts on the specimens after 3 and 24 h were studied using scanning electron microscopy (SEM). Specimens were washed with PBS and treated with both 25% and 50% dimethyl sulfoxide (DMSO) for 1 h each, then placed on gelatin-coated plates with 50% DMSO and freeze-fractured with liquid nitrogen. Specimens were deiced with 50% DMSO and washed with PBS, then macerated for 3 days in osmium liquid (0.1%) at 20°C. Finally, specimens underwent conductive staining, dehydration with graded ethanol, critical-point drying, evaporation coating with tetraosmium, and observation with SEM.

Alkaline phosphatase assay

Osteoblasts were seeded at a density of 3500 cells/cm^2 and were cultured with alpha modified Eagle's medium (Sigma) containing 10% fetal bovine serum (Gibco), 1% 200 mM L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) for 7 days. The cells were washed twice with PBS, scraped off into 0.3 mL of 0.5% Nonidet P-40 (polypxyethylene-9-octyiphenyl ether) containing 1 mM of $MgCl_2$ and 10 mM of Tris(hydroxymethyl)aminomethane (pH 7.5), and sonicated twice for 15 s each with a sonicator (Model W-220, Wakenyaku, Kyoto, Japan). Alkaline phosphatase (ALP) activity was assayed following the method of Kind-King with a test kit (Labo Assay ALP, Wako). Light absorbance of the specimens was measured spectrophotometrically at 405 nm using a microplate reader (VersaMax, Molecular Device Japan, Tokyo, Japan). Total ALP activity was calculated from standard absorbance curves.

Quantification of calcium

Osteoblasts were seeded at a density of 3500 cells/cm^2 and cultured with alpha modified Eagle's medium (Sigma) containing 10% fetal bovine serum (Gibco), 1% 200 mM L-glutamine (Gibco, Invitrogen), and 1% penicillin/streptomycin (Gibco)

for 7 days. The osteoblasts were washed twice with PBS. The extracellular matrix of each preparation was treated with iso-octylphenoxy-polyethoxyethanol (Triton X-100, Roche, Uppsala, Sweden). After the prescribed time period, the amount of calcium present in the acidic supernatant was spectrophotometrically quantified using a commercially available kit (Calcium E-test, Wako). The light absorbance of the specimens was measured spectrophotometrically at 610 nm using a microplate reader (VersaMax, Molecular Device Japan). Total calcium was calculated from standard curves of absorbance.

Preparation of ribonucleic acid and real-time reverse transcriptase polymerase chain reaction

Osteoblasts were cultured on alumina ceramic and CNT/alumina composite for 1 or 5 days. RNA of all cells was extracted from osteoblasts following the method of Yamamoto et al. [26] and complementary DNA was synthesized from the RNA using a reverse transcription (RT) device (ReverTra Ace, Toyobo, Osaka, Japan). Then, two-step polymerase chain reaction (PCR) was performed using a real-time PCR detection system (DNA Engine Opticon system, MJ Japan, Tokyo, Japan) with primers (Takara Bio, Shiga, Japan) specific to Runx 2, osteocalcin, or

glyceraldehyde-3-phosphate dehydrogenase. The fold/change ratios between the test specimens were calculated.

Statistical analyses

The numbers of each inflammatory cell in each specimen in the bone implantation study, numeric data of cell attachment, calcium level, ALP activity, and expression levels of Runx2 and osteocalcin for each specimen were statistically analyzed with the Welch's *t*-test. In the osteoblast attachment test, ANOVA was used for three experimental groups. *P* values of 0.05 or less were considered to indicate significance. SPSS14.0J (SPSS Japan, Tokyo, Japan) was used to conduct the analyses.

Results

Characterization of materials

The characteristics and mechanical properties of alumina ceramic and the CNT/alumina composite are shown in **Table 1**. The average surface roughness values of the alumina ceramic and CNT/alumina composite were 4.12 μm and 4.48 μm, respectively, and the average grain sizes were 1.23 ± 0.5 μm and 0.80 ± 0.4 μm, respectively. Relative density was similar for each. Adding CNTs did not improve bending strength, but the fracture toughness of alumina ceramic improved by approximately 20% after combination with CNTs. Young's modulus and Vickers hardness were similar for alumina ceramic and the CNT/alumina composite. CNTs treated with an ultra-fine grinding machine were dispersed homogeneously in the alumina matrix (Figure 1).

Subcutaneous tissue reactions

Alumina ceramic and CNT/alumina composite specimens embedded in the subcutaneous tissue of mice were collected with peripheral tissue after 1 or 4 weeks, stained with HE, and observed using a light microscope. After 1 week, inflammatory cells consisting mainly of lymphocytes were observed around

alumina ceramic specimens; however, severe inflammatory reactions such as the presence of leukocytes or necrosis were not observed. Images of the tissues surrounding CNT/alumina composite specimens were similar to those of alumina ceramic specimens, with comparative inflammatory reactions (Figures 2A & B). The number of inflammatory cell numbers in the surrounding tissue of both compounds was not significantly different (**Table 2**). After 4 weeks, thin fibrous capsules attached to alumina ceramic had been formed, inflammatory cells including neutrophils or lymphocytes were not observed in peripheral regions, and the inflammatory reaction had disappeared. Similar fibrous capsules of comparative thickness formed on the CNT/alumina composite, and no inflammatory reaction in the peripheral tissue was observed (Figures 2C & D). Taken together, these results showed that the tissue compatibility of CNT/alumina composite with subcutaneous tissues was excellent and comparable to that of alumina ceramic.

Bone tissue reactions

Rabbit femurs in which alumina ceramic or CNT/alumina composite specimens were implanted were collected after 12 or 24 weeks, stained with HE, and

observed using a light microscope. At 12 weeks, new bone had formed around the alumina ceramic, and the fibrous capsule between the ceramic and the bone was rarely observed. The tissue images of implanted CNT/alumina composite were similar (Figures 3A, B, E & F). After 24 weeks, the entire circumference of the alumina ceramic specimen had attached to the bone tissue without gaps, and the surrounding bone tissue was normal. Alumina ceramic specimens were completely incorporated into the bone, and the bone defect was repaired. The circumferences of CNT/alumina composite specimens also showed favorable new bone generation, and composite specimens had attached to the bone tissue and become incorporated. Bone defects were completely repaired and tissue images similar to those of the alumina ceramic specimens were observed (Figures 3C, D, G & H). These results showed that the bone tissue compatibility of CNT/alumina composite is comparable to that of alumina ceramic.

In vitro cell attachment

To study cell attachment to the surface of the specimens, we cultured osteoblasts, chondrocytes, fibroblasts, and smooth muscle cells on specimens of alumina ceramic and CNT/alumina composite and stained them with DAPI after 1, 3, 6, 24,

72, and 168 h to count the number of cells attached to the surface of the specimens. The number of attached cells differed depending on cell type: osteoblasts showed the highest attachment, followed by chondrocytes. Fibroblasts and smooth muscle cells showed about 10% attachment compared to osteoblasts and chondrocytes. The number of attached cells increased in all four cell types over time (Figure 4). A significant difference in the number of cells attached to alumina ceramic and that attached to CNT/alumina composite was observed in osteoblasts after 3 h, chondrocytes after 168 h, and smooth muscle cells after 168 h. The number of osteoblasts attached to either alumina ceramic or CNT/alumina composite was statistically larger than that of the standard cell culture plate (Figure 4A).

The attachment status of osteoblasts to the surfaces of alumina ceramic and CNT/alumina composite specimens was observed using SEM after 3 and 24 h. In both specimens, osteoblasts had attached at surface grooves after 3 h (Figure 5A & B). The attachment status of osteoblasts to the CNT/alumina composite surface area at which CNTs were exposed was similar to that at which they were not exposed. After 24 h, osteoblasts had spread on the surface of both specimens and showed normal morphology (Figure 5C & D).

These results show that CNT/alumina ceramic has comparable or more

favorable cell attachment properties, and CNTs at the surface of the implant did not inhibit attachment.

Alkaline phosphatase activity

ALP, an enzyme produced by osteoblasts, is the most common indicator of osteoblast activity. The ALP activity of the osteoblasts attached to the alumina ceramic or CNT/alumina composite specimens after 7 days of culture was measured. The values for alumina ceramic and CNT/alumina composite were 5.56 units/μl and 5.29 units/μl, respectively, showing no significant difference (Figure 6).

Calcium level

The calcium level of the osteoblasts attached to the surface of the alumina ceramic and CNT/alumina composite specimens after 7 days of culture was measured. The mean calcium levels were 9.60 mg/mL and 13.66 mg/mL, respectively. Although osteoblasts attached to the CNT/alumina composite specimen had a higher calcium level, the difference was not significant (Figure 7).

Expression of osteoblastic genes

As an indicator of osteoblast differentiation, Runx2 (a transcription factor that binds to the promoter region of osteocalcin) and osteocalcin (a bone matrix protein) were measured using real-time RT-PCR with total RNA collected 1 or 5 days after the start of the cultures. Runx2, which is a marker of early differentiation, was measured on day 1, and osteocalcin, a marker of late differentiation, was measured on day 5. No significant difference was found in the expression of Runx2 and osteocalcin with CNT/alumina composite and that with alumina ceramic (Figure 8).

Discussion

CNTs are currently undergoing research and development as new structural and conductive materials for applications in various fields [20,21] because they have unique and useful mechanical and electrical characteristics and significantly improve the properties of base materials with which they are combined. Efforts by medical researchers to improve mechanical strength and durability by combining CNTs with existing biomaterials are ongoing [23,27,28]. We have studied the use of CNTs as reinforcements in alumina ceramic products that have been clinically applied in various fields as biomaterials. Reported improvements in the mechanical properties of CNT/alumina composites can be found in the literature [18,29-35]. These studies showed only small improvement of strength, however, owing to the formation of microstructures that occurs when CNTs are dispersed homogeneously in the ceramic matrix. Homogeneous dispersion causes difficulties because CNTs are hydrophobic and have a large intermolecular force; thus, CNT aggregates form easily when CNT is mixed with hydrophilic materials such as alumina. The presence of these aggregates prohibits CNTs from acting as reinforcement, and the mechanical strength of the ceramic is decreased. Dispersing CNTs in ceramics using conventional methods has been difficult

[17,35-38]. In the present study, the amount of added CNTs in the composite is currently as small as 0.4-2.5 wt%; however, the relative density of the composite is similar to that of alumina ceramic, and the fracture toughness of the composite was on average 20% higher (maximum 69% higher) than that of unreinforced alumina ceramic [22]. This improvement of fracture toughness is a great advantage when composites are used as biomaterials.

The most important issues in the clinical application of CNT/alumina composites as biomaterials are biocompatibility and biological safety. Alumina ceramics have a long clinical history and are proven to have excellent biocompatibility and biological safety [6,16]. To our knowledge, no study to date has reported the *in vivo* biocompatibility or bone tissue compatibility of CNT/alumina composites. In the present study, the most basic tests of biocompatibility—subcutaneous implantation and bone implantation—were performed in accordance with the international standard ISO 10993. Male ddY mice, which are widely used for animal studies, were used. Male mice are generally used for bone-related study because bone formation is influenced on female hormone (estrogen). These studies showed that the CNT/alumina composite has biocompatibility and bone tissue compatibility similar to that of

alumina ceramic. Because ceramic materials are frequently used in areas adjacent to bone, long-term bone tissue compatibility tests at 12 and 24 weeks were performed [40,41]. Alumina ceramic combined with CNTs was shown to be stable for long periods *in vivo*. To observe the bone/implant contact region, we prepared thin sections that included the implant after methyl methacrylate fixation using a saw microtome. These sections enabled direct observation of the interface with few artifacts caused by the implant. In clinical applications of CNT/alumina composites to repair bone defects or fix bone fractures, improved strength would be advantageous if it could be achieved while maintaining bone tissue compatibility similar to that of alumina ceramics. In prosthesis applications for arthroplasty adjacent to bone, CNT/alumina composites must also cause no adverse effects to the base materials. Although carcinogenicity and genotoxicity tests must be performed in the future for confirmation, the results of our study, which show that the CNT/alumina composite has biocompatibility and bone tissue compatibility similar to that of alumina ceramics, is favorable and important for clinical applications.

Another important and basic characteristic of biomaterials is cell attachment to the surface of the material. Bachle et al. have studied cell attachment to

ceramic surfaces by culturing osteoblast-like cells on ceramics and reported that surface roughness influenced initial cell attachment and the spread of cells [42]. In contrast, Yamashita et al. have studied attachment of osteoblast-like cells on 2 kinds of ceramics and reported that surface condition had no effect on initial attachment because the specimen surface was covered by chemically stable oxide [43]. Price et al. have made composites of ceramics and carbon nanofibers and reported that the finer surface of the specimens increased the attachment area of osteoblasts, and the number of attached cells increased with an increase in the addition of carbon nanofibers [44,45]. Thus, various arguments have been made regarding the influence of ceramic surface conditions on cell attachment. Our cell attachment test showed that attachment to the CNT/alumina composite was comparable to or better than that to alumina ceramic. Osteoblast attachment was observed visually using SEM because the CNT/alumina composite is likely to be practically applied as a bone-related biomaterial. Osteoblasts were observed to attach in the groove between particles of both materials, and the cytoplasm spread to surrounding regions after 24 h. Differences in cell attachment status in areas in which CNTs were exposed to the surface were not observed in SEM images. The number of attached osteoblasts was significantly larger in both

specimens compared to the standard cell culture plate, suggesting that the attachment characteristics of osteoblasts to alumina ceramic are maintained even when CNT is added. The contribution of proteins—including extracellular matrix proteins, cytoskeletal proteins, integrins, and cadherins—to cell attachment have been elucidated [46,47]. Further investigation of cell attachment is warranted before CNT/alumina composites can be applied clinically; however, improvements in attachment can be expected to occur after adjustments in the number and condition of CNTs on the composite surface. The bone mineralization function of the attached osteoblasts was maintained after the addition of CNTs to alumina because no significant difference in ALP activity or calcium level was found between CNT/alumina composite and alumina ceramic. In addition, the addition of CNT has little influence on the differentiation of osteoblasts because the expression of differentiation markers, Runx2 and osteocalcin, did not differ.

The safety of CNT particles *in vivo* is another important issue in CNT biomaterial applications [23,24]. Although some reports on the safety of CNTs in biomaterials have been published, further detailed study is required [48-50]. During the use of CNT/alumina composites as biomaterials, the possibility of *in vivo* exposure to CNT particles that separate from composites exists depending on

the location and purpose of use—for example, as wear debris from the socket or the head of the stem prosthesis for total hip arthroplasty. The number of CNTs released from CNT/alumina composites *in vivo* is expected to be very small, and the possibility of adverse effects from CNT particles themselves is low. The majority of clinical applications of CNTs as biomaterials are likely to be as composites rather than CNTs alone, and such applications are expected to be realized in the near future. The safety of each CNT composite will be studied as will the individual safety of CNT particles. Considering the significant advantages of applying CNT composites as biomaterials, the biological safety evaluation of CNT/alumina composites performed in this study may be an important basis for the clinical application of various CNT composites that are likely to emerge in the future.

Conclusions

To investigate basic biocompatibility of a newly developed CNT/alumina composite, we performed a subcutaneous implantation study, a bone implantation study, and a cell attachment test. The results showed biocompatibility and bone tissue compatibility similar to those of alumina ceramic. Provided that CNT/alumina composites have biological safety comparable to that of alumina ceramics, these composites, which have improved mechanical characteristics, could be clinically applied as novel, highly functional biomaterials. The range of ceramic applications could potentially spread to treatments in which the use of ceramics is currently limited owing to brittleness—e.g., artificial joints or bone plates. The CNT/alumina composite developed in this study has great potential to enhance the progress of treatments in various medical fields, and this study pushes the development of clinical applications a step forward.

Future perspective:

CNT/alumina composites, novel materials in which CNTs are combined as reinforcement for alumina ceramic as base material, were developed to improve mechanical properties of alumina ceramic while preserving its basic advantage, and succeeded in remarkable improvement of fracture toughness. Results of in vivo and in vitro study showed similar biocompatibility and bone tissue compatibility compared to alumina ceramics. Applications of CNT/alumina composites as biomaterials that contact to bone, such as prostheses in arthroplasty and devices for bone repair, are especially expected in near future.

Executive summary

CNT/alumina composite

- CNTs were combined with alumina ceramics, and fracture toughness was significantly improved.

Biocompatibility and bone tissue compatibility *in vivo* and *in vitro*

- Biocompatibility and bone tissue compatibility of CNT/alumina composites established through subcutaneous implantation, bone implantation, and cell attachment studies in accordance with ISO10993 are reported.
- The results of basic safety tests showed biocompatibility and bone tissue compatibility similar to those of alumina ceramic.

Conclusions

- With the rapid progress of nanoscience, the advantages of applying nanomaterials such as CNTs in medicine are becoming obvious, and the pursuit of such applications is becoming a cutting-edge research field.
- In actual application, composite materials will be used in most cases rather than the nanoparticles alone, hastening the time from development to clinical application.
- In the application of nanomaterial composites, biological safety tests of the composites will be performed in reference to this study.

Acknowledgements

This research was supported by the CLUSTER program of the Ministry of Education, Culture, Sports, Science and Technology of Japan; by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by the Research and Development of Nanodevices for Practical Utilization of Nanotechnology of the New Energy and Industrial Technology Development Organization of Japan; and by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Bibliography

Papers of special note have been highlighted as:

* = of interest

** = of considerable interest

1. Wang W, Ouyang Y, Pho CK: Orthopaedic Implant Technology: Biomaterials from past to Future. *Ann. Acad. Med. Singapore*. 40, 237–44(2011).
2. Rohr SM, Li MG, Nilsson KG, Nivbrant B: Very low wear of nonremelted highly cross-linked polyethylene cups: an RSA study lating up to 6 years. *Acta Orthop*. 78, 739–45(2007).
3. Puleo DA, Nanci A: Underdtanding and controlling the bone-implant interface. *Biomaterials.* 20, 2311–21(1999).
4. Faghihi S, Zhilyaev AP, Szpunar JA, *et al.*: Nanostructuring of a titanium material by high-pressure torsion improves pre-osteoblast attachment. *Adv. Mater.* 19, 1069–73(2007).
5. Ammar A. : Tissue Compatibility of Different Inttracranial Implant Mterials: In-vivo and In-vitro Studies. *Acta Neurochirurgica*. 72, 45–59(1984).

6. Kress AM, Schmidt R, Holzwarth U, *et al.*: Excellent results with cementless total hip arthroplasty and alumina-on-alumina pairing: minimum ten-year follow-up. *Int. Orthop.* 35, 195–200 (2011). ** Recent research on the alumina-on-alumina prosthesis for total hip arthroplasty.
7. Layton D: A critical appraisal of the survival and complication rates of tooth-supported all-ceramic and metal-ceramic fixed dental prostheses: the application of evidence-based dentistry. *Int. J. Prosthodont.* 24, 417–427 (2011).
8. Boutin P, Christel P, Dorlot JM, *et al.*: The use of dense alumina-alumina ceramic combination in total hip replacement. *J. Biomed. Mater. Res.* 22, 1203–1232 (1988).
9. Bal BS, Greenberg DD, Buhrmester L, Aleto TJ.: Primary TKA with a zirconia ceramic femoral component. *J. Knee Surg.* 19, 89–93 (2006).
10. Ohgushi H, Kotobuki N, Funaoka H, *et al.*: Tissue engineered ceramic artificial joint-ex vivo osteogenic differentiation of patient mesenchymal cells on total ankle joints for treatment of osteoarthritis. *Biomaterials* 26, 4654–4661 (2005).

11. Ueda K, Oba S, Omiya Y, Okada M.: Cranial-bone defects with depression deformity treated with ceramic implants and free-flap transfers. *Br. J. Plast. Surg.* 54, 403–408 (2001).

12. Wang JK, Lai PC, Liao SL.: Late Exposure of the bioceramic orbital implant. *Am. J. Opthalmol.* 147, 162–170 (2009).

13. Raigrodski AJ, Chiche GJ, Potiket N, *et al.*: The efficacy of posterior three-unit zirconium-oxide–based ceramic fixed partial dental prostheses: a prospective clinical pilot study. *J. Prosthet. Dent.* 96, 237–244 (2006).

14. Pirker A, Kocher A.: Immediate, non-submerged, root-analogue zirconia implants placed into single-rooted extraction sockets: 2-year follow-up of a clinical study. *J. Oral. Maxillofac. Surg.* 38, 1127–1132 (2009).

15. Bassim MK, Buss E, Clark MS, *et al.*: MED-EL Combi40 + Cochlear implantation in adults. *Laryngoscope* 115, 1568–1573 (2005).

16. Krell A, Klimake J.: Effects of the homogeneity of particle coordination on solid-state sintering of transparent alumina. *J. Am. Ceram. Soc.* 89, 1985–1992 (2006).

17. Affatato S, Traina F, Toni A.: Microseparation and stripe wear in alumina-on-alumina hip implants. *Int. J. Artif .Organs.* 34, 506–12 (2011).

18. Yamamoto G, Omori M, Hashida T, Kimura H.: A novel structure for carbon nanotube reinforced alumina composites with improved mechanical properties. *Nanotechnology.* 19, 315708 (2008).

19. Kohal RJ, Wolkewitz M, Mueller C.: Alumina-reinforced zirconia implants: survival rate and fracture strength in a masticatory simulation trial. *Clin. Oral. Implants. Res.* 21, 1345–1352 (2010).

20. Iijima S.: Helical microtubules of graphitic carbon. *Nature* 354, 56–58 (1991).

21. Oberlin A, Endo M, Koyama T.: Filamentous growth of carbon through benzene decomposition. *J. Cryst. Growth.* 32, 335–349 (1976).

22. Ueda N, Yamakami T, Yamaguchi T, *et al.*: Fabrication and mechanical properties of high-dispersion-treated carbon nanofiber/alumina composite. *J. Ceram. Soc. Japan* 118, 847–854 (2010). * Shows fabrication of uniformly-dispersed CNT-alumina composites with enhanced mechanical properties.

23. Saito N, Usui Y, Aoki K, *et al.*: Carbon nanotubes: biomaterial applications. *Chem. Soc. Rev.* 38, 1897–1903 (2009). * Recent review on the biomaterial applications of CNTs.

24. Kostarelos K, Bianco A, Prato M.: Promises, facts and challenges for carbon nanotubes in imaging and therapeutics. *Nat. Nanotechnol.* 4, 627–633 (2009).

25. Webster TJ, Siegel RW, Bizions R.: Osteoblast adhesion on nanophase ceramics. *Biomaterials.* 20, 1221–1227 (1999).

26. Yamamoto Y, Udagawa N, Matsuura S, *et al.*: Osteoblasts Provide a Suitable Microenvironment for the Actin of Receptor Activator of Nuclear Factor-κB Ligand. *Endocrinology*. 147, 3366–74(2006).

27. Saito N, Usui Y, Aoki K, *et al.*: Carbon nanotubes for biomaterials in contact with bone. *Curr. Med. Chem.* 15, 523–527 (2008).

28. Usui Y, Aoki K, Narita N, *et al.*: Carbon nanotubes with high bone-tissue compatibility and bone-formation acceleration effects. *Small.* 4, 240–246 (2008).

29. Zanello L, Zhao B, Hu H, Haddon R.: Bone cell proliferation on carbon nanotubes. *Nano. Lett.* 6, 562–567 (2006).

30. Hu H, Ni Y, Montana V, *et al.*: Chemically functionalized carbon nanotubes as substrates for neuronal growth. *Nano. Lett.* 4, 507–511 (2004).

31. Zhan GD, Kuntz JD, Wan J, Mukherjee AK.: Single-wall carbon nanotubes as attractive toughning agents in alumina-baced nanocomposites. *Nat. Mat.* 2, 38–42 (2003).

32. Sun J, Gao L, Jin X.: Reinforcement of matrix with multi-walled carbon nanotubes. *Ceram. Int.* 31, 893–96 (2005).

33. Corral EL, Cesarano J, Shyam A, *et al.*: Engineered nanostructures for multifunctional single-walled carbon nanotube reinforced silicon nitride nanocomposites. *J. Am. Ceram. Soc.* 91, 3129–3137 (2008).

34. Wang X, Padture NP, Tanaka H.: Contact-damage-resistant ceramic/single-wall carbon nanotubes and ceramic/graphite composites. *Nat. Mater.* 3, 539–544 (2004).

35. Carrero-Sanchez JC: Biocompatibility and toxicological studies of carbon nanotubes doped with nitrogen. *Nano. Lett.* 6, 1609–1616 (2006).

36. Estili M, Kawasaki A.: An approach to mass-producing individually alumina-decorated multi-walled carbon nanotubes with optimized and controlled compositions. *Scripta. Mater.* 58, 906–909 (2008).

37. Estili M, Kawasaki A, Sakamoto H, Mekuchi Y, Kuno M, Tsukada T: The homogeneous dispersion of surfactantless, slightly disordered, crystalline,

multiwalled carbon nanotubes in α-alumina ceramics for structural reinforcement. *Acta. Mater.* 56, 4070-4079 (2008).

38. Zhu YF, Shi L, Liang J, *et al.*: Synthesis of zirconia nanoparticles on carbon nanotubes and their potential for enhancing the fracture toughness of alumina ceramics. *Compos. Pt. B. Eng.* 39, 1136–1141 (2008).
39. Wei T, Fan Z, Luo G, Wei F.: A new structure for multi-walled carbon nanotubes reinforced alumina nanocomposite with high strength and toughness. *Mater. Lett.* 62, 641–64 (2008).
40. Lu JX, Gallur A, Flautre B, *et al.*: Comparative study of tissue reactons to calcium phosphate ceramics among cancellous, cortical, and medullar bone site in rabbits. *J Biomed Mater Res.* 42, 357–67(1998).
41. Hayashi K, Matsuguchi K, Uenoyama K, *et al.*: Re-evaluation of the biocompatibility of bionert ceramics in vivo. *Biomaterials.* 13, 195–200(1992).
42. Bachle M, Butz F, Hubner U, *et al.*: Behavior of CAL72 osteoblast-like cells cultured on zirconia ceramics with different surface topographies. *Clin. Oral. Implants. Res.* 18, 53–59 (2007).
43. Yamashita D, Machigashita M, Miyamoto M, *et al.*: Effect of surface roughness on initial responses of osteoblast-like cells on two types of zirconia.

Dent. Mater. J. 28, 461–470 (2009). ** Recent research on the effect of surface roughness on the initial attachment of osteoblast-like cells on ceramics.

44. Price RL, Haberstoh KM, Webster TJ.: Enhanced functions of osteoblasts on nanostructured surfaces of carbon and alumina. *Med. Biol. Eng. Comput.* 41, 372–375 (2003).

45. Price RL, Ellison K, Haberstroh KM, Webster TJ.: Nanometer surface roughness increases select osteoblast adhesion on carbon nanofiber compacts. *J. Biomater. Res.* 70, 129–138 (2004).

46. Webster TJ, Ergun C, Doremus RH, *et al.*: Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *J. Biomed. Mater. Res.* 51, 475–483 (2005).

47. Anselme K.: Osteoblast adhesion on biomaterials. *Biomaterials* 21, 667–681 (2000).

48. Bai Y, Zhang Y, Zhang J, *et al.*: Repeated administrations of carbon nanotubes in male mice cause reversible testis damage without affecting fertility. *Nat. Nanotechnol.* 5, 683–689 (2010).

49. Schipper ML, Nakayama-Ratchford N, Davis CR, *et al.*: A pilot toxicology study of single-walled carbon nanotubes in a small sample of mice. *Nat. Nanotechnol.* 3, 216–221 (2008).

50. Liu Z, Davis C, Cai W, *et al.*: Circulation and long-term fate of functionalized, biocompatible single-walled carbon nanotubes in mice probed by raman spectroscopy. *Proc. Natl. Acad. Sci. USA*. 105, 1410–1415 (2008).

Figure captions

Figure 1. Scanning electron microscopy images of alumina ceramic and carbon nanotube (CNT)/alumina composite

(A) Fracture surface of alumina ceramic treated with hot isostatic pressing (HIP).

(B) Fracture surface of HIP-treated CNT/alumina composite, with 0.8 wt% CNTs. CNTs were highly dispersed in the alumina matrix. Scale bar: 1 μm. Arrow: CNTs.

Figure 2. Tissue images around alumina ceramic and carbon nanotube (CNT)/alumina composite specimens embedded in the subcutaneous tissue of mice **(A)** After 1 week, a mild gathering of inflammatory cells, mainly lymphocytes, was observed around the alumina ceramic specimen. **(B)** After 1 week, the surroundings of the CNT/alumina composite specimen showed similar mild inflammatory reactions. **(C)** After 4 weeks, a thin fibrous capsule was formed and attached to the alumina ceramic specimen, and the inflammatory reaction had disappeared. **(D)** After 4 weeks, a similar fibrous capsule was formed with the CNT/alumina composite, and inflammatory reactions were not observed. Stained with hematoxylin and eosin. Scale bar: 100 μm. * The gap that sample was present.

Figure 3. Tissue images of rabbit femurs into which alumina ceramic or carbon nanotube (CNT)/alumina composite specimens were embedded

Upper panel shows the entire area in which the specimen was embedded **(A-D, 40×)**. Lower panel shows an enlarged image of the border between the specimen and the bone **(E-H, 200×)**. **(A, E)** After 12 weeks, new bone had formed around the alumina ceramic specimen. Fibrous capsules were absent. **(B, F)** After 12 weeks, the image of the tissue around the CNT/alumina composite specimen was similar to that of alumina ceramic. **(C, G)** After 24 weeks, the bone defects were completely repaired. New bone tissue was attached to the alumina ceramic specimen. Arrows indicate regions outside of the bone; the entire specimen was surrounded by bone tissue. **(D, H)** Twenty-four weeks after the implantation of the CNT/alumina composite specimen, bone defects were completely repaired as with alumina ceramic, and bone tissue was attached to the specimen. Stained with hematoxylin and eosin. Scale bar: 100 μm.

Figure 4. Cell attachment to surfaces of an alumina ceramic and a carbon nanotube (CNT)/alumina composite

Time course of the number of cells attached to the surface of the specimens: **(A)** osteoblasts, **(B)** chondrocytes, **(C)** fibroblasts, and **(D)** smooth muscle cells. **(A)** The number of osteoblasts attached to the alumina ceramic and CNT/alumina composite specimens was significantly larger than that in the standard cell culture ($P < 0.05$). **(A-D)** All cell types showed increased attachment over time. The number of attached osteoblasts and chondrocytes was approximately 10 times larger than that fibroblasts and smooth muscle cells. A significant difference in the number of attached cells between alumina ceramic specimens and CNT/alumina composite specimens was observed in osteoblasts after 3 h, chondrocytes after 168 h, and smooth muscle cells after 168 h. $*P < 0.05$ compared to alumina composite.

Figure 5. Scanning electron microscopy images of cell attachment
The surfaces of alumina ceramic **(A)** and carbon nanotube (CNT)/alumina composite **(B)** specimens after 3 h of culture. In both specimens, osteoblasts largely attached to grooves on the specimen surface. The surfaces of alumina ceramic **(C)** and CNT/alumina composite **(D)** specimens after 24 h of culture. Osteoblasts are spread on the surface of both specimens and showed the

morphology of normal osteoblasts. * Spreading osteoblast. Arrow indicates CNT. Scale bar: 5 μm.

Figure 6. Alkaline phosphatase (ALP) activity of osteoblasts

Alkaline phosphatase activity was comparable in carbon nanotubes (CNT)/alumina composite and alumina ceramics specimens on day 7.

Figure 7. Calcium level of osteoblasts

Carbon nanotube (CNT)/alumina composite showed higher values than those of alumina ceramics, but the difference was not significant after 7 days of culture.

Figure 8. Expression of osteoblastic genes

Runx2, a marker of early differentiation was measured at day 1 and osteocalcin, a marker of late differentiation, was measured at day 5 in osteoblasts that were cultured and attached to carbon nanotube (CNT)/alumina composite and alumina ceramic specimens using real-time reverse transcriptase polymerase chain reaction. No significant difference in the expression of Runx2 and osteocalcin occurred between the specimens.

Table 1

Relative density, mechanical properties, surface roughness, and average alumina grain size of alumina ceramic and the CNT/alumina composite

	Sintering method	Sintering temp. and time	Surface roughness [μm]	Alumina grain size [μm]	Relative density [%]	Young's modulus [Gpa]	Vickers hardness [GPa]	Fracture toughness [$MPam^{0.5}$]	Bending strength [MPa]
Alumina	PLA* +HIP	1300°C 2.0 h	4.12	1.23 ± 0.5	99.6	382	21.3 ± 0.3	3.5 ± 0.1	1079 ± 69
CNT/alumina	PLV** +HIP	1350°C 0.5 h	4.48	0.80 ± 0.4	99.6	383	19.9 ± 0.4	4.2 ± 0.3	578 ± 81

* Pressureless sintering in air.

** Pressureless sintering under vacuum.

CNT, carbon nanotube; HIP, hot isostatic pressing.

Table 2

Number of inflammatory cell in subcutaneous tissue 1 week after implantation of carbon nanotube (CNT)/alumina composite and alumina ceramic specimens

	Lymphocytes	Plasma cells	Neutrophils	Macrophages
Alumina*	22.6±7.4	6.2±1.1	6.0±1.9	3.4±2.7
CNT/alumina*	14.4±4.7	10.2±2.3	5.4±2.2	2.5±1.6
P value	0.37	0.15	0.78	0.35
Statistical difference	No significant difference	No significant difference	No significant difference	No significant difference

* Mean ± standard deviation

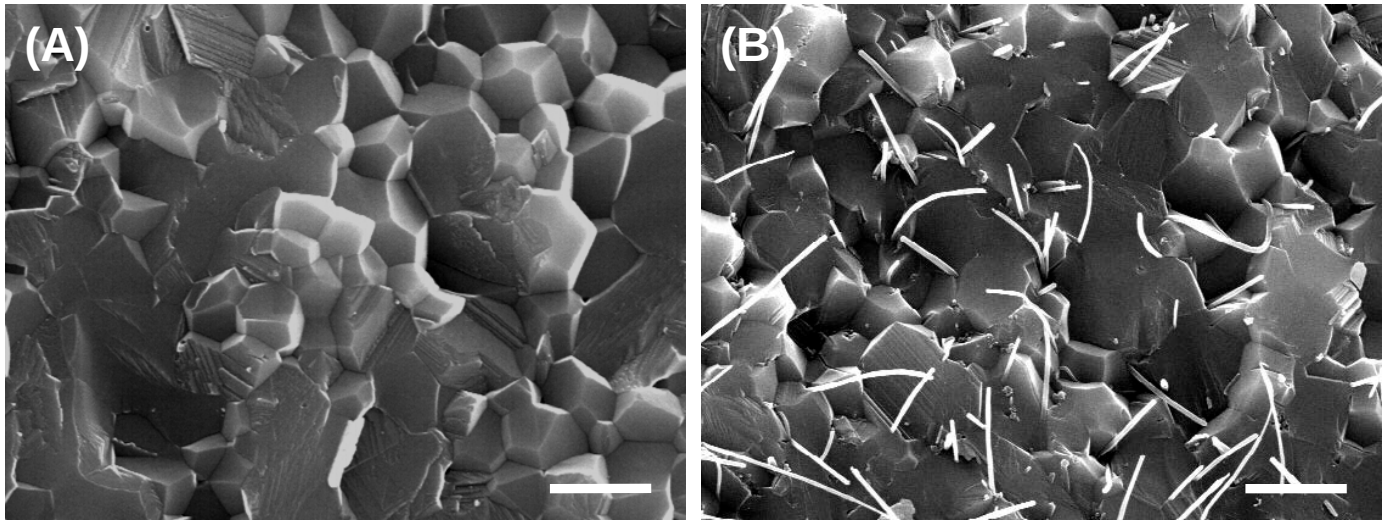


Figure 1

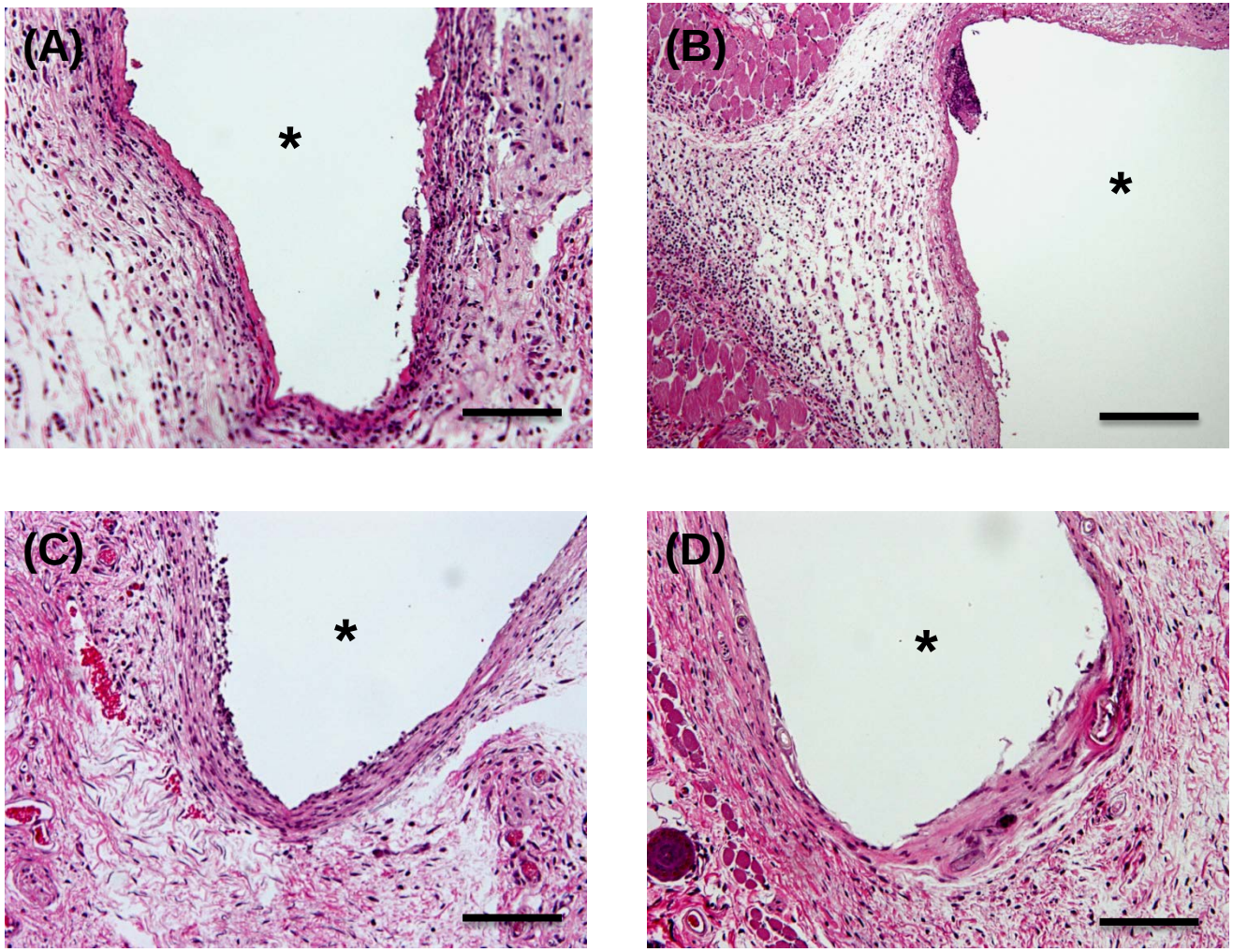


Figure 2

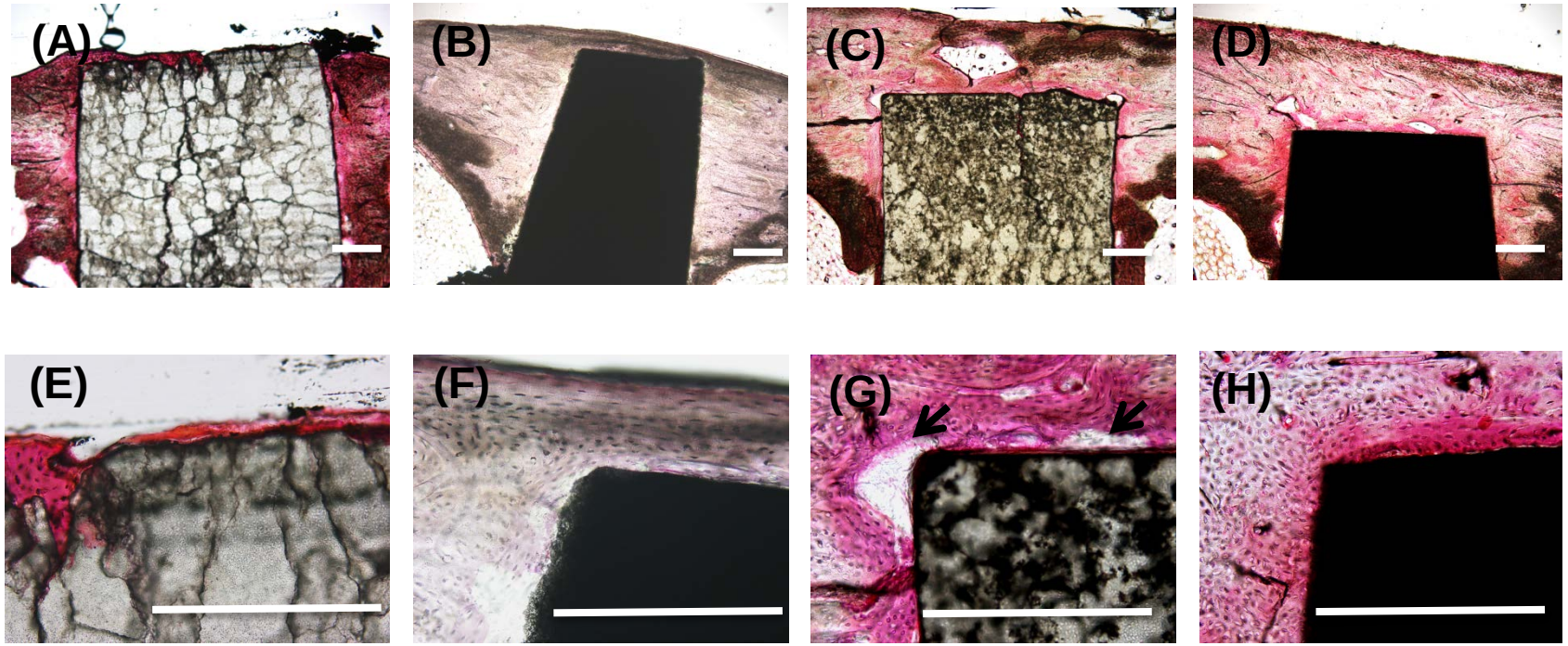


Figure 3

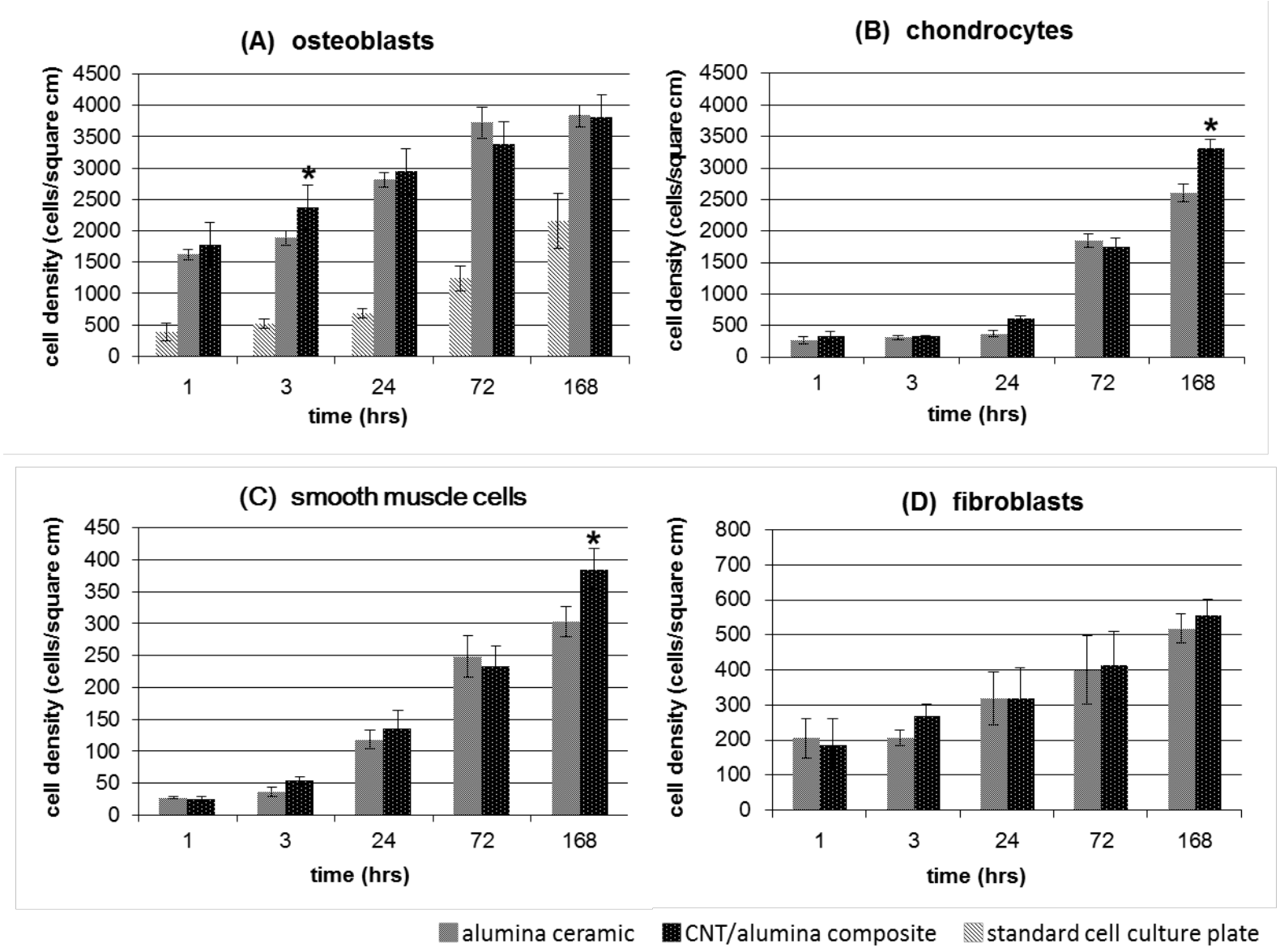

(A) osteoblasts
cell density (cells/square cm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
3
24
72
168
time (hrs)
*
(B) chondrocytes
cell density (cells/square cm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
3
24
72
168
time (hrs)
*
(C) smooth muscle cells
cell density (cells/square cm)
450
400
350
300
250
200
150
100
50
0
1
3
24
72
168
time (hrs)
*
(D) fibroblasts
cell density (cells/square cm)
800
700
600
500
400
300
200
100
0
1
3
24
72
168
time (hrs)
alumina ceramic
CNT/alumina composite
standard cell culture plate

Figure 4

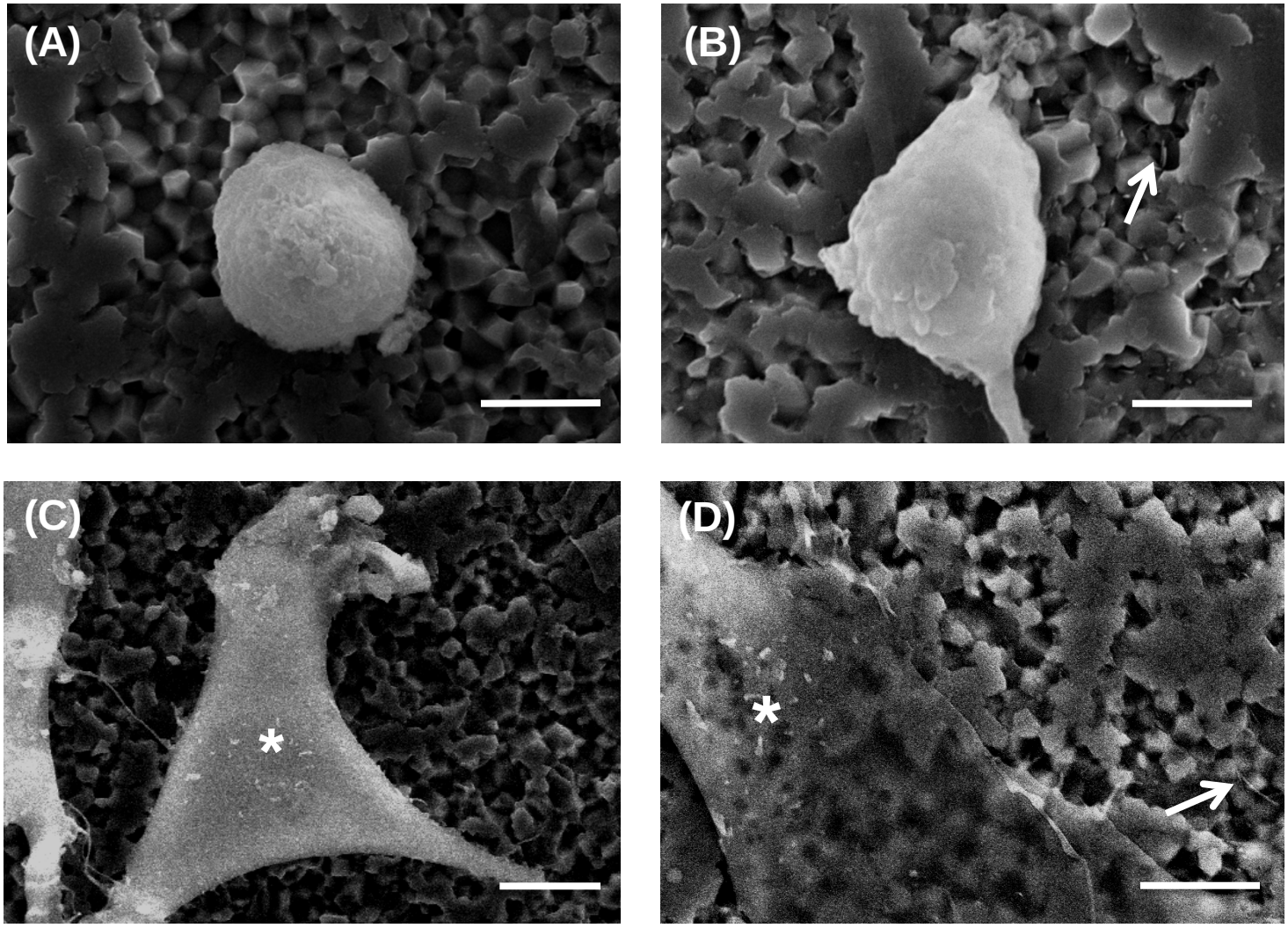


Figure 5

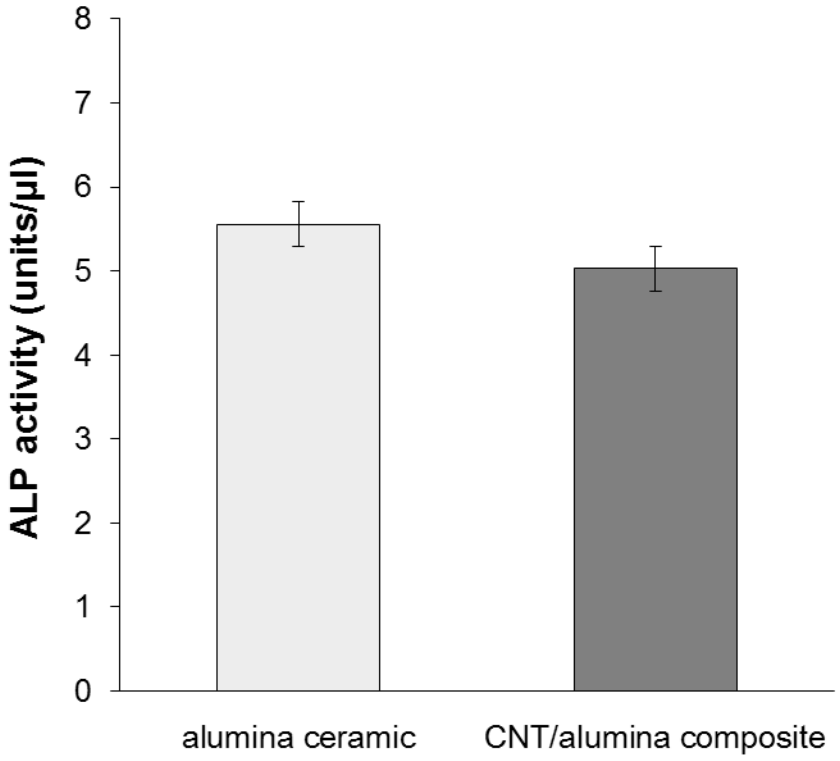

ALP activity (units/µl)
8
7
6
5
4
3
2
1
0
alumina ceramic
CNT/alumina composite

Figure 6

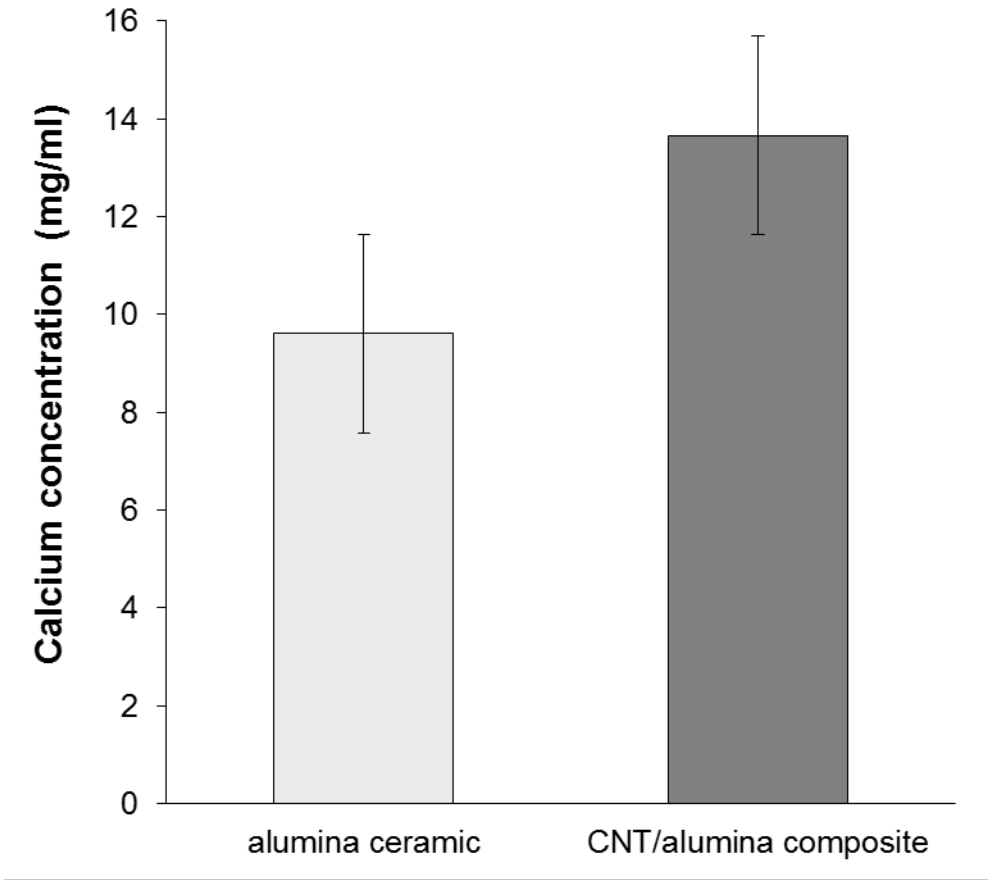
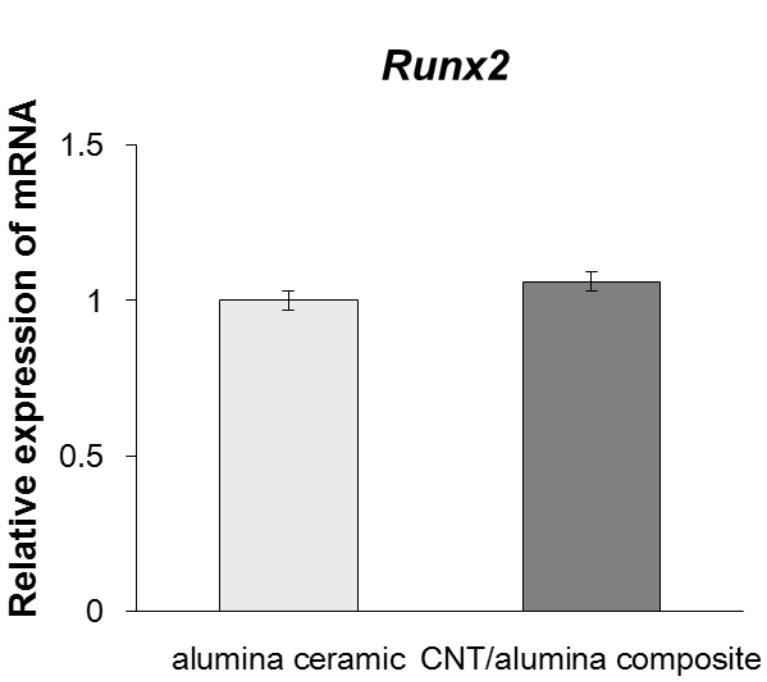

Calcium concentration (mg/ml)
16
14
12
10
8
6
4
2
0
alumina ceramic
CNT/alumina composite

Figure 7


Runx2
Relative expression of mRNA
1.5
1
0.5
0
alumina ceramic
CNT/alumina composite

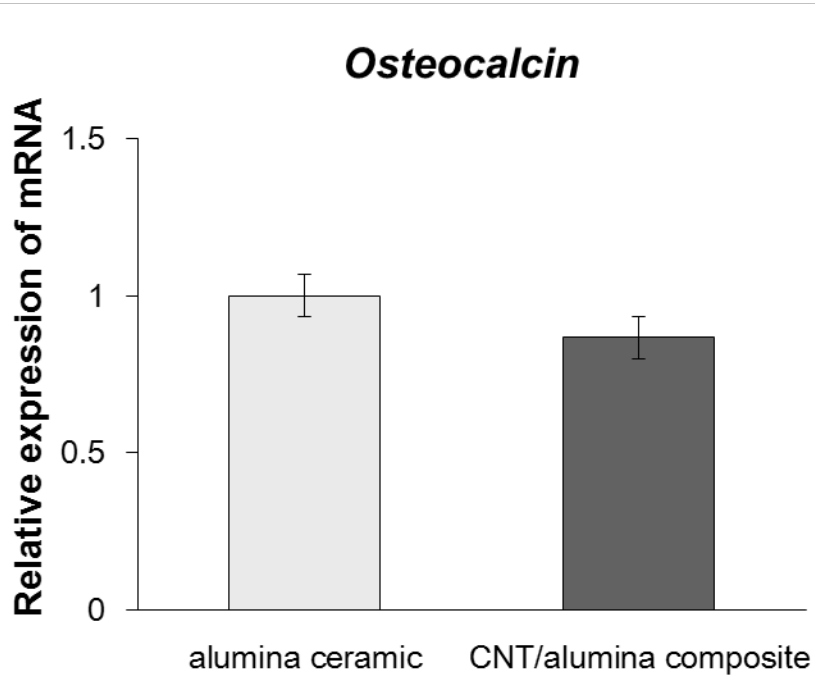

Osteocalcin
Relative expression of mRNA
1.5
1
0.5
0
alumina ceramic
CNT/alumina composite

Figure 8