

Estrogenic Activity of Phthalate Esters by *In Vitro* VTG Assay Using Primary-cultured *Xenopus* Hepatocytes

Yuji NOMURA¹, Naoko MITSUI², Ujjal Kumar BHAWAL³, Masahiko SAWAJIRI⁴, Osamu TOOI², Toru TAKAHASHI⁵ and Masayuki OKAZAKI¹

¹Department of Biomaterials Science, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan

²Biotechnology Research Laboratory, Towa Kagaku Co. Ltd., Hiroshima, Japan

³Research and Development, Dentsply-Sankin KK, Tokyo, Japan

⁴Hiroshima University Hospital, Hiroshima, Japan

⁵Department of Medical Technology, Kumamoto Health Science University, Kumamoto, Japan

Corresponding author, Yuji Nomura E-mail: ynomura@hiroshima-u.ac.jp

Received March 27, 2006/Accepted June 16, 2006

Estrogenic activity of phthalate esters in dental soft resins was evaluated with an amphibian system consisting of a vitellogenin (VTG)-detecting Enzyme-Linked Immunosorbent Assay and a primary-cultured hepatocyte assay using adult male *Xenopus laevis*. In particular, phthalate esters – Di-n-butyl phthalate (DBP), Butyl phthalyl butyl glycolate (BPBG), Benzyl butyl phthalate (BBP), and Benzyl benzoate (BB) – were investigated. Bisphenol A (BPA) was prepared for comparison with these chemicals, and 17 β -estradiol (E2) was used as a positive control. The chemicals were diluted in dimethyl sulfoxide (DMSO) to obtain final concentrations ranging from 10⁻¹¹ to 10⁻⁴ mol/l. BPA induced estrogenic activity at a concentration of 1.1 \times 10⁻⁶ mol/l, while E2 showed at 4.1 \times 10⁻¹¹ mol/l. DBP, BBP, BB, and BPBG showed no estrogenic activity at concentrations between 4 \times 10⁻⁷ mol/l and 1 \times 10⁻⁴ mol/l. The latter result indicated that these phthalate esters might be metabolically transformed into non-estrogenic substances in *Xenopus* hepatocytes. Furthermore, this study demonstrated that through *in vitro* metabolism assessment, the estrogenic activity of chemical substances could be directly detected in terms of VTG secretion in primary-cultured *Xenopus* hepatocytes.

Key words: Sandwich ELISA, Estrogenic activity, Phthalate ester, Dental soft resin

INTRODUCTION

Soft resins are widely used for short-term temporary restorations after cavity preparation and as denture lining materials in dentistry¹⁾. Generally, they are imparted flexibility by a plasticizer additive²⁾. The most available plasticizer in dentistry are the phthalate esters³⁾, largely because of their very low toxicity. However, recent environmental toxicology researches expressed concern that some phthalate esters were identified as EDCs that indicate estrogenicity^{4–6)}.

Exposure to EDCs with hormone- or antihormone-like activity is one possible cause for the alleged decline in male reproductive health in humans and the increase in reproductive deficits in wildlife^{7,8)}. Biological effects of EDCs may occur at a lower concentration as compared with that required to induce cytotoxicity effects on local irritation. Against this background, it is feared that EDCs may have a detrimental effect on the living systems even with the ingestion of a very small quantity.

There is growing concern that phthalate esters may cause endocrine disruption – as indicated by some bioassays; however, the results are not conclusive yet. In some previous studies, the authors reported on the estrogenic activity of phthalate esters by MCF-7 cell proliferation assay, receptor binding assays, reporter gene expression assays using cul-

tured cells and yeast cells, and a yeast two-hybrid assay^{9–13)}. Despite the usefulness of these methods in detecting estrogenicity, they have their limitations too.

For the detection and characterization of environmental chemicals with potential to disrupt the endocrine system, *in vivo* bioassays are developed by the Organization for Economic Co-operation and Development (OECD) Task Force on Endocrine Disrupters Testing and Assessment. On the other hand, *in vitro* tests are also play an important role because they can rapidly identify suspect compounds and most certainly, reduce the number of test animals. In this connection, risk assessment of EDCs has gradually been conducted in screening tests that used yeast and bacteria, such as estrogen receptor competitive binding assay and reporter gene assay based on estrogen-responsive promoters¹⁴⁾.

However, these *in vitro* assays – as screening tests – lack *in vivo* metabolites. For instance, environmental compounds may be metabolically transformed into estrogenic or non-estrogenic substances in the body or cells. Therefore, it is also necessary to examine the direct effects of these compounds on animals or cells in the screening tests.

With regard to the abovementioned point, *in vitro* primary hepatocyte assay detecting hepatic vitellogenin (VTG) as a biomarker might be useful

for screening the estrogenic and anti-estrogenic activities of chemicals. Recently, yolk precursor protein VTG has been widely used as an estrogenic biomarker. It is synthesized in hepatocytes and induced directly by estrogenic compounds. Thus, through *in vitro* assessment of metabolism in cultured primary hepatocytes, the biological activities of estrogens and xenoestrogens are well exemplified by detecting the synthesis of VTG in male oviparous vertebrates^{15,16}.

We have proposed VTG as an ideal biomarker in the screening of estrogenic activities of endocrine disrupters in a wide range of oviparous animals¹⁷. In the present study, we evaluated the estrogenic activity of phthalate esters in dental soft resins with an amphibian system consisting of VTG-detecting ELISA and primary-cultured hepatocyte assay using adult male *Xenopus laevis*.

MATERIALS AND METHODS

Specimens

For standard solutions, Di-n-butyl phthalate (DBP), Butyl phthalyl butyl glycolate (BPPG), Benzyl butyl phthalate (BBP), and Benzyl benzoate (BB) were purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. Bisphenol A (BPA; Kanto Chemical Co., Tokyo, Japan) and 17 β -estradiol (E2; Sigma-Aldrich Inc., St. Louis, MO, USA) were also investigated. The chemicals were diluted in dimethyl sulfoxide (DMSO; Kanto Chemical Co., Tokyo, Japan) to obtain the experimental concentrations.

Animals

Adult male *X. laevis* frogs were supplied by Yamamura Frog Store (Hiroshima, Japan). The animals were kept at 22°C under a 12:12-hour light-dark cycle, and fed with *Xenopus* No. 3 (Oriental Yeast Co. Ltd., Tokyo, Japan) three times per week.

Primary culture

Hepatocytes were isolated from adult male *X. laevis* as described by Kawahara *et al.*¹⁶. A one-step perfusion method was used as follows. The liver was perfused with 100-200 ml of a perfusion solution (pH 7.4) containing 0.55% NaCl, 0.014% KCl, 1 mmol/l pyruvate, 0.05% glucose, 0.5% BSA, 10 mmol/l HEPES, and 0.1% collagenase (collagenase, Wako Pure Chemicals Co.). The perfused livers (about 2.5 g) were minced and incubated in the perfusion solution at 25°C for 15 minutes with gentle shaking. Cell suspension was then sieved through a nylon mesh. Following which, the cells were washed four times with the culture medium by low-speed centrifugation at 300 rpm/min for 1-2 minutes, which eliminated the non-parenchymal cells almost totally from the final preparation (usually more than 95% liver parenchymal cells). The cells (4×10^4 cells) were then

inoculated into 96-well tissue culture plates (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) and incubated in air at 22°C. The culture medium consisted of 50% Leibovitz L-15 medium which contained 1 μ g/ml insulin, 10 nmol/l dexamethasone, 0.05% glucose, and antibiotics (50 U/ml of penicillin and 50 μ g/ml of streptomycin).

After two days of preculture, the cells were exposed to estrogen by replacing the culture medium with an estrogen-containing medium. Estrogens to be tested were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 0.1 and 1 mmol/l and used after appropriate dilution with the culture medium (not to exceed 0.1% DMSO). 0.1% DMSO was used as a control. Various concentrations of phthalate esters in conjunction with physiologically relevant concentrations of E2 and BPA were tested. In brief, all chemicals were serially three-fold diluted in culture medium. The concentrations used were: 1×10^{-8} to 1.4×10^{-11} mol/l for E2, 1×10^{-5} to 4.1×10^{-8} mol/l for BPA, and 1×10^{-4} to 4.1×10^{-7} mol/l for the four phthalate esters. Culture medium was renewed every three days by replacing 2/3 volume of the medium. At the end of culture period, the culture media were harvested into 96-well microtiter plates.

Sandwich ELISA

VTG level in each culture medium was measured using *Xenopus* VTG ELISA Kit (Japan EnviroChemicals Ltd., Osaka, Japan). Albumin (ALB) in culture medium was measured with ELISA assay, according to our previous report¹⁷. Reactions were carried out at room temperature, and absorbance of reactions was determined at 450 nm with a microplate reader (Spectra Fluor, Tecan, Salzburg, Austria). Assay range of the standard curves was 0.25-50 ng/ml for VTG and 0.25-250 ng/ml for ALB. VTG concentration in culture medium was calculated from the linear part of the VTG standard curve. Culture media were diluted at least twice for ELISA assay.

Statistical analysis

Statistical analysis was performed using the software package SPSS 11.5J (SPSS Inc., Chicago, USA). VTG concentration in culture medium was expressed as mean \pm standard deviation of the mean for three replicate wells within the same isolation. Data were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison tests. Differences were considered significant at $p < 0.05$.

RESULTS

Typical ELISA standard curves used to quantify *Xenopus* VTG and ALB

Assay range of the standard curves was 0.25-50 ng/

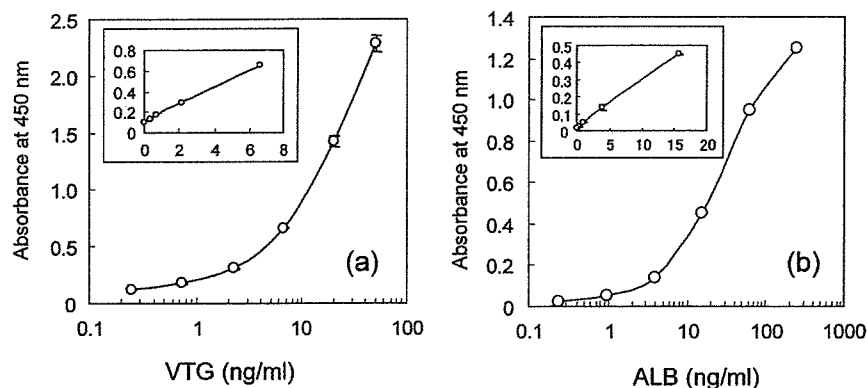


Fig. 1 Standard curves of ELISA for quantifying (a) VTG and (b) ALB. The inserts show locally magnified curves. Each value indicates mean \pm S.D. ($n=3$).

ml for VTG and 0.25-250 ng/ml for ALB (Fig. 1). A linear increase (0.2-6 ng/ml) in the ELISA reaction was observed for VTG concentrations. These ELISA standard curves were used as the calibration curves for the chemicals tested. Culture media were diluted at least twice for ELISA assay. Therefore, the quantitation limit of VTG in the culture medium was 0.5 ng/ml.

Dose-response curves of BPA-dependent VTG and ALB induction

Dose-response curves of BPA-dependent VTG and ALB induction were compared to those of E2 (Fig. 2). VTG level of control was 0.53 ± 0.06 ng/ml. BPA showed induction of VTG at a concentration of 1.1×10^{-6} mol/l (induced VTG level: 1.62 ng/ml), while E2 showed at 4.1×10^{-11} mol/l (induced VTG level: 3.91 ng/ml). Estimated values for the potency relative to E2 were obtained using the concentrations of E2 and BPA required for inducing 1 ng/ml VTG. It was estimated as 0.003% for BPA when E2 was 100% (Table 1).

Dose-response curves of phthalate esters

Dose-response curves of phthalate ester (DBP, BPBG, BBP, BB)-dependent VTG and ALB induction are shown in Fig. 3. All phthalate esters showed no estrogenic activity at concentrations between 4×10^{-7} mol/l and 1×10^{-4} mol/l. Further, judging from

ALB induction, all phthalate esters showed no signs of toxicity to hepatocyte within the tested concentration range. Estrogenic potency relative to 100% E2 was estimated to be under 0.00001% for each compound (Table 1).

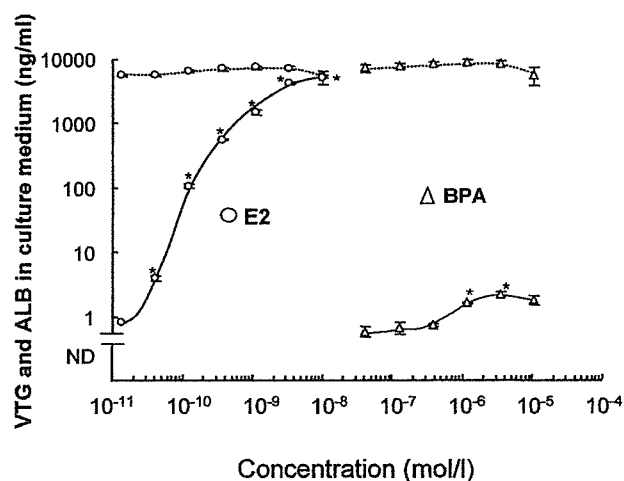


Fig. 2 Dose-response curves of BPA-dependent VTG (solid line) and ALB (dotted line) induction, compared with the results for E2. *: significant difference compared to the control ($p < 0.05$). ND: < 0.5 ng/ml. Each value indicates mean \pm S.D. ($n=3$).

Table 1 Estrogenic potential of phthalate esters relative to E2, through induction of VTG synthesis

Compound	LOEC (M) ^a	Relative potency to E2 (%) ^b
17 β -estradiol (E2)	4.1×10^{-11}	100
Bisphenol A (BPA)	1.1×10^{-6}	0.003
Dibutyl phthalate (DBP)	$> 1.0 \times 10^{-4}$	< 0.00001
Butyl phthayl butyl glycolate (BPBG)	$> 1.0 \times 10^{-4}$	< 0.00001
Benzyl butyl phthalate (BBP)	$> 1.0 \times 10^{-4}$	< 0.00001
Benzyl benzoate (BB)	$> 1.0 \times 10^{-4}$	< 0.00001

^aLOEC is defined as lowest effective concentration among concentrations tested in this study.

^bRelative potency was the ratio between concentrations of E2 and BPA required to induce 1 ng/ml VTG in culture medium.

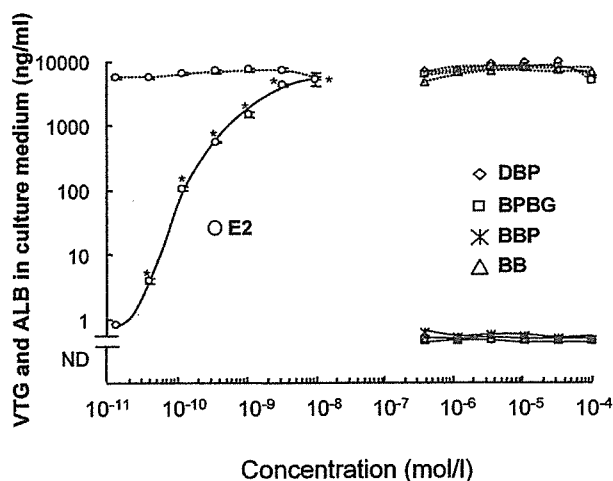


Fig. 3 Dose-response curves of phthalate ester-dependent VTG (solid line) and ALB (dotted line) induction, compared with the results for E2. *: significant difference compared to the control ($p < 0.05$). ND: < 0.5 ng/ml. Each value indicates mean \pm S.D. ($n=3$).

DISCUSSION

Various technologies have been used to screen for EDCs — namely, MCF-7 cell proliferation assay, receptor binding assays, reporter gene expression assays using cultured cells and yeast cells, and a yeast two-hybrid assay⁹⁻¹³. For example, Hashimoto *et al.*¹⁰ reported that BPA and BBP induced estrogenic activity at a concentration of 5×10^{-5} mol/l in a reporter gene assay (yeast two-hybrid system) and an estrogen/estrogen receptor (ER- α) competition binding assay (fluorescence polarization system). On the other hand, Harris *et al.*¹² reported that BBP and DBP showed weak estrogenic activity using a recombinant yeast screen for mitogenic effects on estrogen-responsive human breast cancer cells. BBP was estimated to be 1,000,000-fold less potent than E2. Likewise, Nishihara *et al.*¹³ reported that BBP induced estrogenic activity at a concentration of 5×10^{-4} mol/l by yeast two-hybrid system, and was estimated to be 1,700,000-fold less potent than E2.

In the present study using *Xenopus* hepatocytes, the lowest effective concentration of E2 was 4×10^{-11} mol/l. Therefore, BBP was expected to induce VTG at a concentration around 1×10^{-5} mol/l. However, in parallel with DBP, BB, and BPBG, BBP showed no estrogenic activity at concentrations between 4×10^{-7} mol/l and 1×10^{-4} mol/l, while BPA showed weak activity at 1×10^{-6} mol/l. The results obtained in this study might mean that BBP was metabolized to non-estrogenic metabolites by *Xenopus* hepatocytes. In another report from NIES (National Institute of Environmental Studies, <http://www.nies.go.jp/edc/estrogen/>), when BBP was tested by *in vitro* yeast

two-hybrid system in absence of rat liver S9 mix, it was estimated to be 130,000-fold less potent than E2; then, in the presence of rat liver S9 mix, BBP did not indicate any estrogenic activity. Taken together, the present result from *Xenopus* hepatocyte assay was in good agreement with the result obtained from yeast two-hybrid system in the presence of rat liver S9 mix.

On the other hand, Picard *et al.*⁹ reported that while BBP — as a parent compound — stimulated MCF-7 proliferation in the E-Screen assay (at 10^{-6} mol/l up to 10^{-5} mol/l), none of its metabolites — which were identified in the culture medium — had estrogenic activities. They also reported that only 10% of the initial BBP remained in the culture medium after BBP was extensively metabolized by MCF-7 cells. By aligning our results with this report⁹, our data might indicate that *Xenopus* hepatocytes have a high ability to metabolize xenobiotics, or that BBP has no estrogenic effects on *Xenopus laevis*. Concerning this assumption, it is necessary to know the binding activity of BBP to *Xenopus* estrogen receptor, although studies using competitive assays have found evidence that BBP does bind to the estrogen receptor in other species (rabbits, rats, and trouts)^{14,18,19}.

To evaluate the effects of chemicals using *in vitro* bioassays with cultured cells, it is important to provide evidence of practical viability through exposure testing. In this study, *Xenopus* hepatocytes were used. This was because *Xenopus laevis* is well established as an experimental animal and it is easy to prepare hepatocytes. Additionally, amphibians hold a peculiar ecological position because they experience both water and land environments in their life history. Indeed, the emergence of amphibians has generated new momentum for animal models. Their advantages as alternative experimental animals include developed four limbs, conversion of respiratory system from bronchial type to pulmonary type, and conversion of excretion from ammonia to urea/uric acid. Therefore, amphibians are considered to be a precious species as an environmental indicator among vertebrates.

In conclusion, *Xenopus* hepatocyte assay can directly estimate the estrogenic activity of phthalate esters in dental soft resins, whereby no estrogenic activities were shown in this study. Absence of estrogenic activity indicated that phthalate esters might be metabolically transformed into non-estrogenic substances in hepatocytes. To confirm the assumption, further study is needed to estimate the binding affinity of these phthalate esters to *Xenopus* estrogen receptor.

ACKNOWLEDGEMENTS

This work was supported by the fund for endocrine

disrupters from the Ministry of the Environment, Japan.

REFERENCES

- 1) Abadie FR. Plastic stopping – Update. *J Prosthet Dent* 1979; 42: 470-476.
- 2) Arai T, Kobayashi H. Effect of plasticizers on setting properties of temporary restorative resin. *Japan J Conserv Dent* 1996; 39: 573-586.
- 3) Murata H, McCabe JF, Jepson NJ, Hamada T. The influence of immersion solutions on the viscoelasticity of temporary soft lining materials. *Dent Mater* 1996; 12: 19-24.
- 4) Mylchreest E, Wallace DG, Cattley RC, Foster PMD. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicol Sci* 2000; 55: 143-151.
- 5) Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalate esters. *Toxicol Sci* 1998; 46: 282-293.
- 6) Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Lucier GW, Jackson RJ, Brock JW. Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 2000; 108: 979-982.
- 7) Shultz VD, Phillips S, Sar M, Foster PM, Gaido KW. Altered gene profiles in fetal rat testes after in utero exposure to di(n-butyl) phthalate. *Toxicol Sci* 2001; 64: 233-242.
- 8) Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jégou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Müller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE. Male reproductive health and environmental xenoestrogens. *Environ Health Perspect* 1996; 104: 741-803.
- 9) Picard K, Lhuguenot JC, Lavier-Canivenc MC, Chagnon MC. Estrogenic activity and metabolism of n-butyl benzyl phthalate *in vitro*: Identification of the active molecule(s). *Toxicol Appl Pharmacol* 2001; 172: 108-118.
- 10) Hashimoto Y, Moriguchi Y, Oshima H, Nishikawa J, Nishihara T, Nakamura M. Estrogenic activity of chemicals for dental and similar use *in vitro*. *J Mater Sci Mater Med*. 2000; 11: 465-468.
- 11) Hashimoto Y, Kawaguchi M, Miyazaki K, Nakamura M. Estrogenic activity of tissue conditioners *in vitro*. *Dent Mater* 2003; 19: 341-346.
- 12) Harris CA, Henttu P, Parker MG, Sumpter JP. The estrogenic activity of phthalate esters *in vitro*. *Environ Health Perspect* 1997; 105: 802-811.
- 13) Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, Takatori S, Kitagawa Y, Hori S, Utumi H. Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J Health Sci* 2000; 46: 282-298.
- 14) Zacharewski T. Identification and assessment of endocrine disruptors: Limitations of *in vivo* and *in vitro* assays. *Environ Health Perspect* 2000; 108: 577-582.
- 15) Sumpter JP, Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ Health Perspect* 1995; 103: 173-178.
- 16) Kawahara A, Sato K, Amano M. Regulation of protein synthesis by estradiol-17 β , dexamethasone, and insulin in primary cultured *Xenopus* liver parenchymal cells. *Exp Cell Res* 1983; 148: 423-436.
- 17) Mitsui N, Tooi O, Kawahara A. Sandwich ELISAs for quantification of *Xenopus laevis* vitellogenin and albumin and their application to measurement of estradiol-17 β effects on whole animals and primary-cultured hepatocytes. *Comp Biochem Physiol C Toxicol Pharmacol* 2003; 135: 305-313.
- 18) Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 1995; 103: 582-587.
- 19) Andersen HR, Andersson AM, Arnold SF, Autrup H, Barfoed M, Beresford NA, Bjerregaard P, Christiansen LB, Gissel B, Hummel R, Jorgensen EB, Korsgaard B, Le Guevel R, Leffers H, McLachlan J, Moller A, Nielsen JB, Olea N, Oles-Karasko A, Pakdel F, Pedersen KL, Perez P, Skakkeboek NE, Sonnenschein C, Soto AM, Sumpter JP, Thorpe SM, Grandjean P. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. *Environ Health Perspect* 1999; 107(Suppl 1): 89-108.

Detailed Consideration of Physicochemical Properties of CO₃apatites as Biomaterials in Relation to Carbonate Content Using ICP, X-ray Diffraction, FT-IR, SEM, and HR-TEM

Rie YOKOTA¹, Hidetaka HAYASHI¹, Isao HIRATA¹, Yasuo MIAKE¹, Takaaki YANAGISAWA² and Masayuki OKAZAKI¹

¹Department of Biomaterials Science, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

²Department of Ultrastructural Science, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
Corresponding author, Masayuki Okazaki E-mail:okazakix@hiroshima-u.ac.jp

Received June 7, 2006/Accepted July 21, 2006

CO₃apatites with different carbonate contents were synthesized at 60±1°C and pH 7.4±0.2 under different carbonate concentrations (0-0.3 mol/L) in the supplied solutions. Their physicochemical properties were analyzed using various methods. Inductively coupled plasma gave accurate chemical analysis data for calcium and phosphate contents. X-ray diffraction analysis showed a clear chemical shift at high carbonate content. A CO₃²⁻ absorption peak area approximately proportional to carbonate content was observed through Fourier transmission infrared spectroscopy. Scanning electron microscopy and high-resolution transmission electron microscopy revealed a dramatic change of the crystal shape. Osteoblast proliferation at the surface of each CO₃apatite-collagen sponge indicated that osteoblasts deformed to expand and cover the surface of the sponge, and appeared to adhere well to the sponge.

Key words : CO₃apatites, Carbonate contents, Detailed analyses

INTRODUCTION

An inorganic substance of human hard tissue is CO₃apatite, which contains many trace elements such as Mg²⁺, Fe²⁺, Zn²⁺, Na⁺, CO₃²⁻, HPO₄²⁻, F⁻, Cl⁻, etc., in addition to Ca²⁺, PO₄³⁻, and OH⁻ as the main components^{1,2}. In particular, enamel apatites contain approximately 1-3 wt% of carbonate, whereas dentin and bone contain approximately 3-5 wt% of carbonate³. In the same vein, the crystallinity of teeth and bone are quite different. Enamel apatite is highly crystallized in contrast to dentin and bone, which are poorly crystallized. At this juncture, it should be mentioned that carbonate content and crystallinity seem to be related to the caries susceptibility^{4,5} of enamel and the resorbability of bone during daily metabolism⁶.

Carbonate interferes with the crystallization of apatites and exerts a weakening effect on the bonds in the apatite structure. Due to these influences, the solubility of dental apatites containing carbonate increases – and hence the susceptibility to caries⁷. For synthesized CO₃apatites, their crystal features are dramatically changed^{8,9}. The apparent solubility of CO₃apatites over pH 4-8 at 37°C is related to their crystallinity. In particular, the degree of increase in the solubility of CO₃apatites having high crystallinity changes greatly in the region of 0-3 wt% of carbonate content – which approximates to that of enamel apatites. On the other hand, bone apatites have a higher carbonate content than enamel apatites and

make a composite with 30-40 wt% of collagen.

To date, many researchers¹⁰⁻¹² have reported on various kinds of material to be used as biomaterials – and all studies were conducted with duly sufficient attention to biocompatibility. However, the concept of delving into the compositions of biological hard tissues was not necessarily reflected during the development of these biomaterials. On this note about biomaterial composition, we have synthesized a CO₃apatite (CO₃Ap) with a chemical composition and crystallinity similar to bone at pH 7.4 and 60°C^{13,14}. CO₃Ap powder was mixed with a collagen solution, of which the antigenicity was removed by enzymatic treatment, and formed into CO₃Ap-collagen pellets. After insolubilization by UV irradiation, the composites showed remarkably reduced disintegration and maintained their shape. Further, they showed good biocompatibility when implanted beneath the periosteum cranii of rats.

With recent advances and current developments in tissue engineering¹⁵⁻²⁰, porous hard tissue biomaterials are expected²¹. To invade the inner core, these materials need a much larger pore size than osteoblasts – with an average diameter of approximately 10 μm and without taking into account the deformation and projection length of osteoblasts. This is because osteoblasts sometimes deform with their expanded projections and become less likely to invade the deeper core by adhering to the walls of pores. Due to this mandatory requirement of large pore size, CO₃Ap was mixed with a neutralized

collagen gel whereby CO₃Ap-collagen mixtures with different CO₃Ap contents and porosity were lyophilized into sponges^{22,23}. SEM observation of CO₃Ap-collagen sponges showed favorable pores for cell invasion, with pore size ranging from 50 to 300 μ m. Mouse osteoblast MC3T3-E1 cells were cultured in α MEM with 10% FCS for two weeks. Hematoxylin-eosin staining confirmed that osteoblast cells invaded well into the CO₃Ap-collagen sponge.

However, to date, there is still no detailed consideration pertaining to the carbonate content of CO₃apatites as biomaterials. While there are reports on the crystallinity and solubility of carbonate apatites with different carbonate contents, the detailed role of carbonates for bone metabolism has not been fully clarified. Therefore, there is an urgent and pressing need to clarify the physicochemical properties of carbonate apatites. Fortunately, with the development of advanced chemical analytical tools, detailed analyses can now be performed more accurately and then compared with previous reports.

As a first step of our research into the carbonate content of CO₃apatites, we examined – in this study – the physicochemical properties of synthesized CO₃apatites in detail, using inductively coupled plasma (ICP), X-ray diffraction, FT-IR, SEM, and HR-TEM to obtain an index of carbonate apatites as hard tissue biomaterials.

MATERIALS AND METHODS

Synthesis of CO₃apatites

Hydroxyapatite (HAp) and CO₃apatites with five different carbonate contents (CO₃Ap_{0.005}, CO₃Ap_{0.01}, CO₃Ap_{0.03}, CO₃Ap_{0.06}, CO₃Ap_{0.3}) were synthesized at 60 \pm 1°C and pH 7.4 \pm 0.2. A 0.5 L solution of 0.2 mol/L Ca(CH₃COO)₂·H₂O and a 0.5 L solution of 0.12 mol/L NH₄H₂PO₄ containing 0, 0.005, 0.01, 0.03, 0.06, and 0.3 mol/L (NH₄)₂CO₃ were added to a mechanically stirred solution of 1.3 mol/L acetate buffer. The suspensions were stirred for three hours and then kept at room temperature for a day. CO₃Aps were then separated by filtration, washed with distilled water, and dried at 60°C. The samples were synthesized in duplicate.

Identification by X-ray diffraction, FT-IR and chemical analysis

X-ray diffraction was employed to identify precipitates and estimate the degree of crystallinity. Measurements were done with a Shimadzu X-ray diffractometer (DX1, Shimadzu Co. Ltd., Kyoto, Japan) with graphite-monochromatized CuK α radiation at 30 kV and 30 mA in a continuous scan mode (4° 2 θ /min and 0.5° 2 θ /min). FT-IR analysis was carried out with a Shimadzu spectrometer (FT-IR 8400S, Shimadzu Co. Ltd., Kyoto, Japan) by diffuse reflectance method using powder samples containing

each apatite (concentration: 1 mg/100 mg KBr) with number of scans at 100.

Fifty milligrams of each CO₃Ap was dissolved completely in 0.1 N HCl solution. Calcium and phosphate concentrations (n=5) were determined using an inductively coupled plasma (ICP) analyzer (SPS7800ICP, SII NanoTechnology Inc., Tokyo, Japan). ICP multi-element standard solution (XSTC-22, SPEX CertiPrep Inc., Metuchen, NJ, USA) was used for calibration. Each 10 mg of CO₃Ap sample (n=5) was taken into a Conway dish, and carbonate concentrations were determined by the titration method as described by Conway²⁴.

SEM and HR-TEM observations

Scanning electron micrographs (SEM) of crystals were obtained with a Hitachi instrument (S-4300, Hitachi Co. Ltd., Tokyo, Japan). HR-TEM observation was also carried out at high magnification. Each sample was embedded in epoxy resin, then sectioned very thinly (about 60 nm) with a microtome equipped with a diamond knife. These thin samples were put into the TEM holder and observed with a Topcon high-resolution transmission electron microscope (EM002B, Topcon Co. Ltd., Tokyo, Japan) at 200 kV.

Preparation of CO₃Ap-collagen sponge

0.5 wt% of calf skin collagen solution (Cellgen, Koken Co. Ltd., Tokyo, Japan), treated by the application of enzymes to minimize antigenicity, was neutralized with 0.1 N NaOH, then mixed immediately with 70 wt% of each CO₃apatite by dry weight. The mixture gels were put into 96-well culture plates. The plates were then frozen at –80°C for two hours and dried in a freeze dry machine (Eyela Co. Ltd., Tokyo, Japan) for 24 hours. The CO₃Ap-collagen sponges were subjected to UV irradiation – by placing them 10 cm from the UV lamp (10 W, 253.7 nm) – for four hours to become insoluble.

Culture of osteoblasts

Mouse osteoblast-like MC3T3-E1 cells derived from untransformed mouse bone marrow were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and maintained in a continuous culture at 37°C in a 5% CO₂ humidified atmosphere. Cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) solution supplemented with 10% heat-inactivated FBS. Penicillin (100 U/ml) and streptomycin (100 μ g/ml) were added to the media. The osteoblast-like cells (2.5 \times 10⁵ cells/well) were grown for one week in α MEM (minimum essential medium alpha modification) with 10% heat-inactivated FBS in 96-well plates containing CO₃Ap-collagen sponges at 37°C in a 5% humidified atmosphere. The culture medium was replaced every three days. The specimens were first fixed by replacing the culture medium with 2%

glutaraldehyde, and then dehydrated in an ethanol-water series (60-100%). Finally, sponges with cells were observed with an SEM (S-4300, Hitachi Co. Ltd., Tokyo, Japan).

RESULTS

The chemical compositions of synthesized CO_3Ap s analyzed using an inductively coupled plasma (ICP) chemical equipment are shown in Table 1. The calcium content of the samples was not significantly different from each other, although it was lower than that of the stoichiometrical 10 mmol/g of theoretical hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Total phosphate content was also lower than the stoichiometrical 6 mmol/g and decreased greatly at high CO_3 content of $\text{CO}_3\text{Ap}_{0.3}$. As for the CO_3 content, it increased gradually and then greatly at high CO_3 content of $\text{CO}_3\text{Ap}_{0.3}$. Total value of phosphate and carbonate contents approached the stoichiometrical phosphate content of 6 mmol/g. However, at higher content, it exceeded the stoichiometrical value. The CO_3 content of $\text{CO}_3\text{Ap}_{0.06}$ (CO_3 content = 0.80 ± 0.07 mmol/g, *i.e.*, 4.8 wt%) was similar to that of bone.

Fig. 1 shows the X-ray diffraction pattern of each CO_3Ap . Crystallinity, which can be evaluated in terms of relative peak intensity and half-value width of the (300) reflection, decreased with increasing CO_3 content as given in previous reports^{7,8}. Even at the lower content of CO_3^{2-} ions, crystallinity decreased greatly. The (300) reflection peak shifted at high CO_3 content (Fig. 2). This meant that most CO_3^{2-} ions were substituted into PO_4^{3-} positions.

FT-IR spectra expressed as percent transmittance became broader over the wave number range of 400-4000 cm^{-1} with increasing CO_3 content (Fig. 3). The FT-IR spectra of CO_3Ap s showed clear CO_3^{2-} ion absorption peaks at 1410-1450 cm^{-1} . The expanded relative area mostly due to CO_3^{2-} ion absorption, when expressed as absorbance, increased with increasing CO_3 content (Fig. 4).

Scanning electron microscopy (SEM) showed that hydroxyapatite had a needle-like crystal feature, and that the crystal feature of CO_3Ap s changed dramatically to become flake-like and broccoli-like as shown in Fig. 5. High-resolution transmission electron microscopy (HR-TEM) of the cross-sectional shape of crystals showed that hydroxyapatite was slender and hexagonal, while CO_3Ap changed gradu-

Table 1 Chemical compositions of precipitates

Sample	Ca (mmol/g)	P (mmol/g)	CO_3 (mmol/g)
HAp	9.01 ± 0.09	5.74 ± 0.09	—
$\text{CO}_3\text{Ap}_{0.005}$	8.45 ± 0.09	5.48 ± 0.07	0.12 ± 0.05
$\text{CO}_3\text{Ap}_{0.01}$	8.49 ± 0.19	5.62 ± 0.12	0.21 ± 0.09
$\text{CO}_3\text{Ap}_{0.03}$	8.72 ± 0.09	5.50 ± 0.11	0.58 ± 0.06
$\text{CO}_3\text{Ap}_{0.06}$	8.78 ± 0.16	5.46 ± 0.15	0.80 ± 0.07
$\text{CO}_3\text{Ap}_{0.3}$	8.56 ± 0.12	4.69 ± 0.11	1.57 ± 0.03

Stoichiometrical compositions of hydroxyapatite
 $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$: Ca=10 mmol/g, P=6 mmol/g

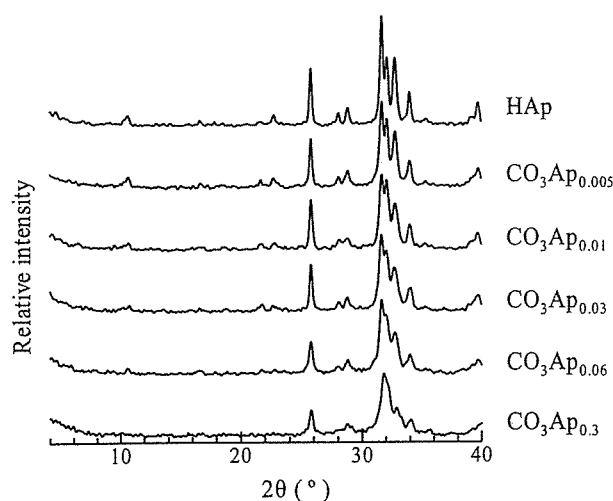


Fig. 1 X-ray diffraction patterns of CO_3Ap s with different CO_3 contents.

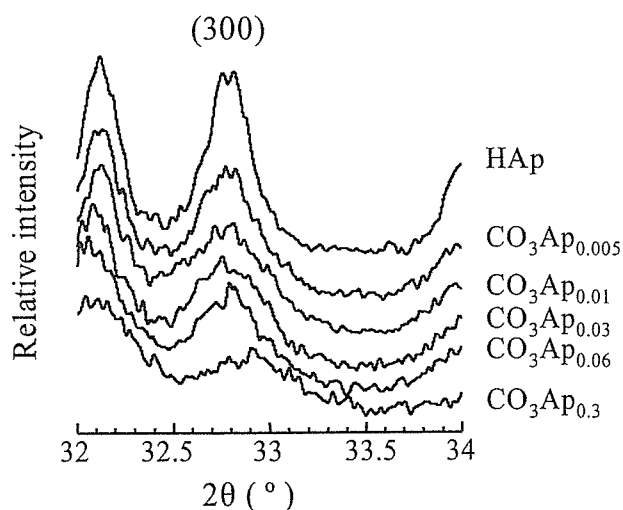


Fig. 2 Expanded X-ray (300) reflection peaks of CO_3Ap s.

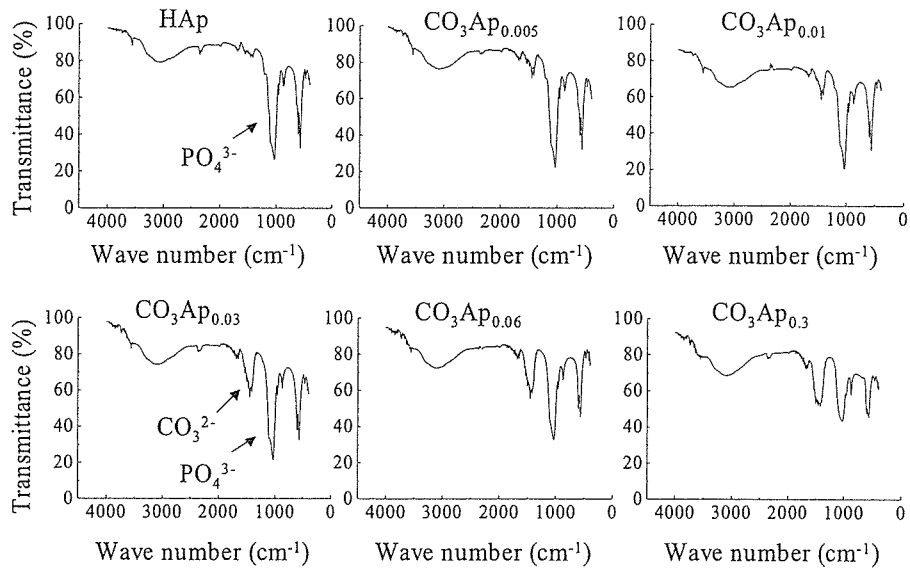


Fig. 3 Fourier transmission infrared spectra of CO₃Aps with different CO₃ contents.

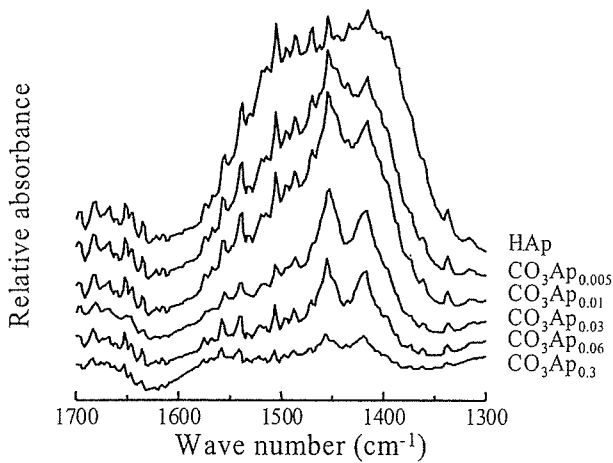


Fig. 4 Expanded FT-IR absorption bands mostly due to CO₃²⁻ ion, of which the peaks can be observed at about 1410-1450 cm⁻¹.

ally from thin to indistinct (Fig. 6). At high magnification, a crystal lattice image and defects were observed.

Fig. 7 shows the SEM photos after one-week incubation of osteoblasts on the surface of each CO₃Ap-collagen sponge. Osteoblasts deformed to expand and cover the sponge sample. They expanded their projections and appeared to adhere well to the sponge surface.

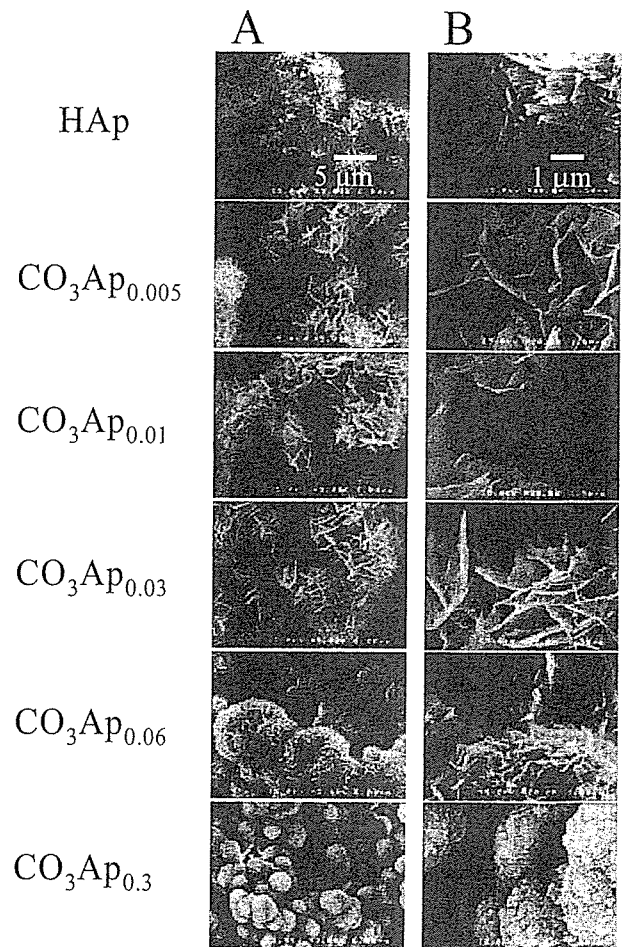


Fig. 5 Scanning electron micrographs of CO₃Aps (A), together with their magnified micrographs of CO₃Aps (B).

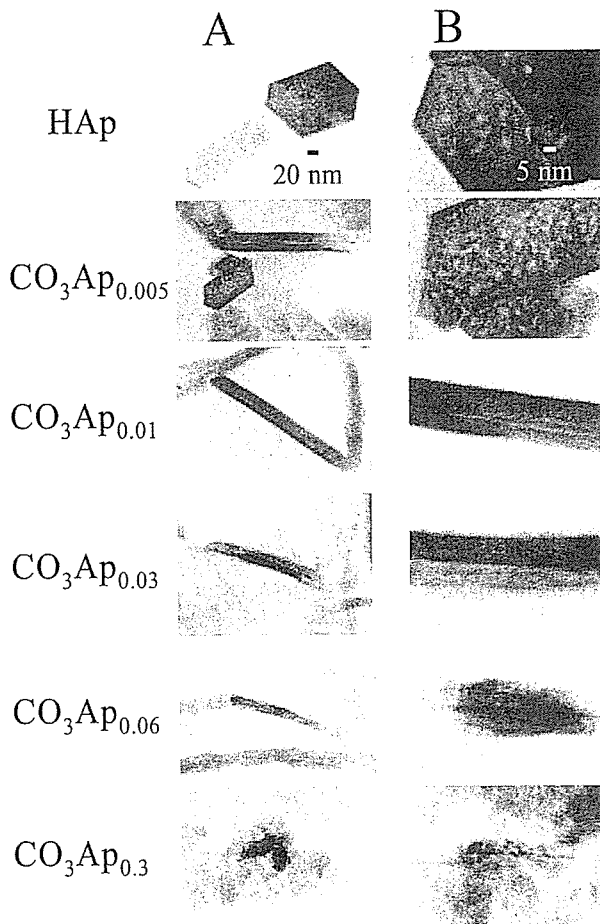


Fig. 6 High-resolution transmission electron micrographs of CO_3Aps (A), together with their magnified micrographs (B).

DISCUSSION

Many researchers have investigated the physico-chemical properties of CO_3Aps ^{4,5,7-9}; however, claims and observations from these researches are yet to be confirmed. Over the past decade, advancements in chemical analytical tools have made more accurate chemical analyses to be possible. Most notably, ICP analysis has been developed to enable detailed analysis to be performed.

In this study, we were able to obtain more reliable data of P concentration. Fortunately too, Ca and P data obtained using ICP were relatively similar to those obtained previously by atomic absorption and spectrophotometry⁸. Calcium and phosphate contents were lower than those of stoichiometrical values due to the formation of Ca- and P-deficient apatites in relation to crystallinity. As for the chemical analysis of carbonate concentration, it still depended on the Conway method²⁴. Although this is a reliable method, it does not provide high accuracy especially at low concentrations. At higher concentrations, P+ CO_3 content exceeded the stoichiometrical value of 6 mmol/g — at which all CO_3^{2-} ions are substituted into PO_4^{3-} positions. A few explanations might be proffered for the obtained P+ CO_3 value, although their effects were very small — namely, partial substitution of CO_3^{2-} into OH^- positions, CO_3^{2-} adsorption on the crystal surface, and/or error of synthesis and chemical analysis.

LeGeros⁷ and Okazaki *et al.*⁸ found that by synthesizing at different temperatures, crystallinity decreased with increasing carbonate content. They also showed that the a-axis dimension decreased with the substitution of CO_3^{2-} ions into PO_4^{3-} positions in the

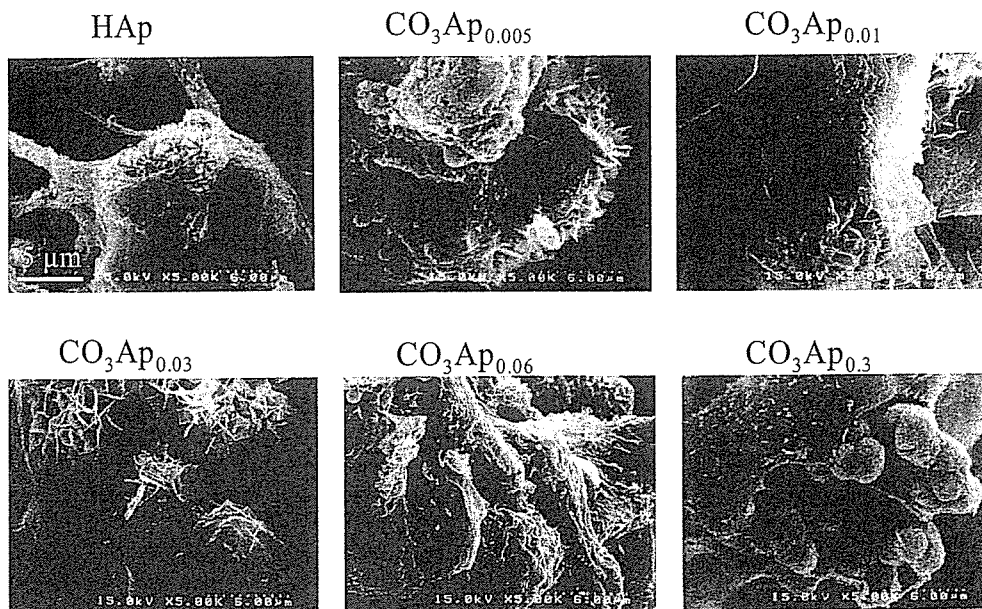


Fig. 7 Scanning electron micrographs of osteoblasts cultured onto CO_3Ap -collagen sponges with different CO_3 contents after 1-wk incubation.

case of apatite crystals synthesized under aqueous conditions, while the c-axis dimension increased with the substitution of CO₃²⁻ ions into OH⁻ positions in the case of apatite crystals synthesized under dry conditions at high temperature⁷⁾. To investigate the crystallinity of biomimetic apatites as biomaterials, apatites were synthesized under aqueous conditions. As a result, a shift of the (300) reflection peak to a high angle direction was recognized, indicating shrinkage of the crystal lattice. Therefore, the substitution of CO₃²⁻ ions into the PO₄³⁻ positions accounted for the shrinkage in the a-axis dimension.

The crystallinity of CO₃apatites synthesized in this study was lower than that of CO₃apatites synthesized at higher temperatures such as 80°C⁷⁻⁹⁾. It was observed that a relatively small amount of CO₃ could significantly affect the physicochemical properties. As shown in Figs. 5 and 6, both crystal size and shape changed dramatically. Disorder or lattice imperfection increased with increased substituting CO₃²⁻ ions. Gradually, it became hard to maintain the hexagonal shape, which became distorted due to the difference between the ionic radius of PO₄³⁻ (0.228 nm) and that of CO₃²⁻ (0.198 nm)²⁵⁾.

In this study, with the purpose of applying large-amount production to biomaterials, the calcium concentration in the supplied solution was much higher than that in previous studies^{8,9)}. Therefore, according to the calcium concentration in the supplied solution, a higher carbonate concentration was also supplied. Consequently, the amount of carbonate content in the apatite crystals differed with each synthesis experiment due to different calcium concentrations in the supplied solutions. In other words, CO₃ contents could not be compared by simply using the CO₃/P ratio due to differences arising from the supplied solutions. In the case of high calcium concentration in the supplied solution, and hence an increase in absolute carbonate concentration, the uptake of CO₃²⁻ ions in the apatite crystal would definitely increase and this uptake is not stoichiometric.

Based on the results obtained in the present study, it could be said that crystallinity and chemical composition significantly influence the applicability of CO₃apatites as biomaterials. This is because crystallinity and chemical composition are important factors during bone regeneration in relation to bone apatite dissolution by osteoclasts and bone formation by osteoblasts. In conclusion, when taking into account the results of chemical analysis and X-ray diffraction, CO₃Ap_{0.06} appeared to be similar to bone and is thus suitable for application as a biomaterial. To further explore its potential as a biomaterial, biological evaluation of CO₃Ap is currently in progress.

ACKNOWLEDGEMENTS

This study was supported in part by Grants-in-aid

for Scientific Research, Nos. 15659465 and 18390515, from the Ministry of Education, Science, Sports and Culture.

REFERENCES

- 1) Miles AEW. Structural and chemical organization of teeth, Vol 2, Academic Press, New York, 1967.
- 2) Van Wazer JR. Phosphorus and compounds, Interscience, New York, 1958.
- 3) Brudevold F, Steadman LT, Smith FA. Inorganic and organic components of tooth structure. Ann NY Acad Sci 1960; 85: 110-132.
- 4) Sobel AE, Shaw JH, Hanok A, Nobel S. Calcification. XXVI. Caries susceptibility in relation to composition of teeth and diet. J Dent Res 1960; 39: 462-472.
- 5) LeGeros RZ. Apatite crystallites: effects of carbonate on morphology. Science 1967; 155: 1409-1411.
- 6) van Furth R. Mononuclear phagocytes: biology of monocytes and macrophages, Kluwer Academic Publisher, Dordrecht, 1992.
- 7) LeGeros RZ. Calcium phosphates in oral biology and medicine. In: Monographs in oral science, Myers HM (ed.), Karger, Basel, 1991.
- 8) Okazaki M, Moriwaki Y, Aoba T, Doi Y, Takahashi J. Solubility behavior of CO₃apatites in relation to crystallinity. Caries Res 1981; 15: 477-483.
- 9) Okazaki M, Takahashi J, Kimura H, Aoba T. Crystallinity, solubility, and dissolution rate behavior of fluoridated CO₃apatites. J Biomed Mater Res 1982; 16: 851-860.
- 10) Hench LL, Splinter RJ, Allen WC, Greelee TK. Bonding mechanisms at the interface of ceramic prosthetic materials. J Biomed Mater Res Symposium 1971; No. 2: 117-141.
- 11) Linkow LI, Chercheve P. Theories and techniques of oral implantology, CV Mosby, St Louise, 1970.
- 12) Oka M, Ushio K, Kumar P, Ikeuchi K, Hyon SH, Nakamura T, Fujita H. Development of artificial cartilage. Eng In Medicine 2000; 214(H): 59-68.
- 13) Okazaki M, Ohmae H, Hino T. Insolubilization of apatite-collagen composites by UV irradiation. Biomaterials 1989; 10: 564-568.
- 14) Okazaki M, Ohmae H, Takahashi J, Kimura H, Sakuda M. Insolubilized properties of UV-irradiated CO₃apatite-collagen composites. Biomaterials 1990; 11: 568-572.
- 15) Constantz BR, Ison IC, Fulmer MT, Poser RD, Smith ST, VanWagoner M, Ross J, Goldstein SA, Jupiter JB, Rosenthal DI. Skeletal repair by *in situ* formation of the mineral phase of bone. Science 1995; 267: 1796-1799.
- 16) Service RF. Bone remodeling and repair (News) — Tissue engineers build new bone. Science 2000; 289: 1498-1500.
- 17) Ignatius AA, Ohnmacht M, Claes LE, Kreidler JP, Palm F. A composite polymer/tricalcium phosphate membrane for guided bone regeneration in maxillofacial surgery. J Biomed Mater Res 2001; 58: 564-569.

- 18) Bokhari M, Birch M, Akay G. Polyhipe polymer: a novel scaffold for *in vitro* bone tissue engineering. *Adv in Exper Med and Biol* 2003; 534: 247-254.
- 19) Yamasaki Y, Yoshida Y, Okazaki M, Shimazu A, Uchida T, Kubo T, Akagawa Y, Hamada Y, Takahashi J, Matsuura N. Synthesis of functionally graded MgCO₃apatite accelerating osteoblast adhesion. *J Biomed Mater Res* 2002; 62: 99-105.
- 20) Yamasaki Y, Yoshida Y, Okazaki M, Shimazu A, Kubo T, Akagawa Y, Uchida T. Action of FGMgCO₃ Ap-collagen composite in promoting bone formation. *Biomaterials* 2003; 24: 4913-4920.
- 21) Ohgushi H, Goldberg VM, Caplan AI. Heterotopic osteogenesis in porous ceramics induced by marrow cells. *J Orthop Res* 1989; 7: 568-578.
- 22) Itoh M, Shimazu A, Hirata I, Yoshida Y, Shintani, H, Okazaki M. Characterization of CO₃Ap-collagen sponges using X-ray high-resolution microtomography. *Biomaterials* 2004; 25: 2577-2583.
- 23) Tieliewuhan Y, Hirata I, Sasaki A, Minagi H, Okazaki M. Osteoblast proliferation behavior and bone formation on and in CO₃apatite-collagen sponges reinforced with a porous hydroxyapatite frame. *Dent Mater J* 2004; 23: 258-264.
- 24) Conway EJ. *Microdiffusion analysis and volumetric error*, 3rd ed, Van Nostrand, New York, 1950.
- 25) Solis-Correa H, Gomez-Lara J. Approximation of spherical polyatomic thermochemical radii of general formula MX_nz. *J Chem Educ* 1987; 64: 942-943.

Photodegradation of Humic Substances on MWCNT/Nanotubular-TiO₂ Composites

Zhiping Zhu,^{1,2} Yi Zhou,² Hongwen Yu,¹ Tomoko Nomura,¹ and Bunshi Fugetsu*^{1,3}

¹Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810

²Department of Chemistry and Environment Engineering, Changsha University of Science and Technology, Changsha 410076, P. R. China

³Creative Research Initiative "Souise," Hokkaido University, Sapporo 001-0021

(Received April 28, 2006; CL-060513; E-mail: hu@ees.hokudai.ac.jp)

We report on the first use of MWCNT/nanotubular-TiO₂ (multiwalled carbon nanotube, MWCNT; TiO₂-derived nanotube, nanotubular-TiO₂) composites as catalysts for photodegradation of aquatic humic substances (HSs). The MWCNT was demonstrated to be capable of enhancing the activity of the photocatalyst. The best degradation efficiency was obtained by using MWCNT/nanotubular-TiO₂ composites having 20% MWCNTs as the photocatalyst.

Elimination of humic substances (HSs) from contaminated water is significant in aquatic system for a number of reasons. First, HSs impart a visible (yellow/brown) color^{1,2} to water; this can cause aesthetic contamination even at very low concentrations. Second, HSs are capable of forming complexes with heavy metal ions³ and/or pollutant organic species⁴ (such as pesticides); this can enhance the absolute solubility and/or the lifetime of these pollutants in water. Moreover, HSs are suspected to be the essential precursors of mutagenic halogenated compounds formed in water after the chlorination.^{5,6}

A number of promising techniques have been established for elimination of HSs from the contaminated water. Physical treatment, such as the conventional coagulation/filtration, adsorption and reverse osmosis method offers simpler approaches to the aquatic HSs.^{7,8} Chemical and biological elimination methods, on the other hands, also find wide range of applications.^{9,10}

Photoelimination, for example, the use of photocatalysts for degrading aquatic HSs, as a typically chemical treatment, is of great interest. However, in case of using titanium dioxide (TiO₂) as the photocatalysts, sufficient amounts of oxygen must be supplied to the reaction system by continuously bubbling the HS/TiO₂ suspension with CO₂-free oxygen.¹¹

In this study, we have established a new type of photocatalyst with which aquatic humic substances (HSs) can be degraded without need for oxygen supply. Multiwalled carbon nanotubes (MWCNTs) were coupled with TiO₂-derived nanotubes (nanotubular-TiO₂) to form MWCNT/nanotubular-TiO₂ composite photocatalysts. To our best knowledge, this is the first report dealing with the use of MWCNT/nanotubular-TiO₂ composite photocatalysts for photoelimination of aquatic HSs.

The nanotubular-TiO₂ was prepared using the TiO₂ nanoparticles as the precursory materials. The TiO₂ nanoparticles were obtained by a sol-gel method^{12,13} using titanium tetrabutoxide Ti[O(CH₂)₃CH₃]₄ as the precursors. The resultant TiO₂ nanoparticles (0.6 g), a certain amount (MWCNT/TiO₂ = 5, 10, 20, and 30 wt %) of highly dispersed MWCNTs (those were obtained by the zwitterionic surfactant dispersion method¹⁴) were introduced into a Teflon-lined stainless steel autoclave containing 10M NaOH. This mixture was then heated in an oven



Figure 1. Typical scanning electron microscope (SEM, Hitachi S4800) images of the resultant MWCNT/nanotubular-TiO₂ composites. Bar full size: 300 nm. MWCNTs = 20%.

at 125 °C for 24 h, similarly to previous reports.^{15,16} After being cooled down to room temperature, they were filtrated (Millipore 0.45 μm membrane filter); washed with 0.1 M aqueous HCl solution and then deionized water until its pH value reached to about 7. The products were dried at 105 °C for 6 h and then were calcined at 350 °C for 8 h in ambient atmosphere.

Scanning electron microscopy (SEM) observations (Figure 1) have demonstrated that the resultant products were consisted of TiO₂-derived nanotubes (nanotubular-TiO₂) together with the highly dispersed MWCNTs. Nanotubular-TiO₂, in general, were built up mainly by amorphous components and hence having poor photocatalysis activities. The MWCNT/nanotubular-TiO₂ composites were further calcined at 350 °C in air, in order to convert nanotubular-TiO₂ into anatase (and hence the higher photocatalysis activities). The successful conversion of the nanotubular-TiO₂ into anatase was demonstrated by XRD analysis of the calcined MWCNT/nanotubular-TiO₂ composites (data not shown). Note that MWCNTs disappeared at the calcination temperature higher than 495 °C.

Model aquatic HS solutions (30 mg/L humic acid; pH was adjusted at 4.0 with ammonia and nitric acid solution) were photolyzed in 0.1% suspensions of the resultant MWCNT/nanotubular-TiO₂ composite catalysts using a mercury lamp. Before the mercury lamp was being switched on, the model aquatic HS/MWCNT/nanotubular-TiO₂ solutions were sonicated for 5 min, magnetically stirred in a dark condition for 30 min, in order to achieve the adsorption-desorption equilibrium. This concentration value (C₀) was used as the beginning concentration after the dark adsorption.¹⁷ During the photodegradation, approximately 3 mL samples were withdrawn regularly from the photo-reactor (every 30 min or 1 h for each sampling). These samples were filtrated with 0.45 μm Millipore membrane filters and measured using a UV-vis spectrophotometer at 436 and 400 nm (indicating color removal), and 365, 280, and 254 nm (representing TOC normalized aromatic moieties). As can be seen from the typical photodegradation experimental data (Figure 2), the MWCNT/nanotubular-TiO₂ composites provided a better pho-

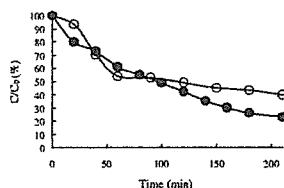


Figure 2. Photodegradation of aquatic humic substances (HSs) with MWCNT/nanotubular-TiO₂ composites (●) and the sole nanotubular-TiO₂ (○) as the photocatalysts. MWCNT = 20%; $\lambda = 254$ nm.

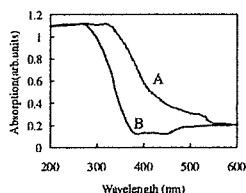


Figure 3. UV-vis absorption spectra of the sole nanotubular-TiO₂ (A) and the MWCNT/nanotubular-TiO₂ composites (B, MWCNT = 20%).

photodegradation coefficient than that of the sole nanotubular-TiO₂ for degradation of the aquatic humic substance (HS). Furthermore, for the MWCNT/nanotubular-TiO₂ composites, a significant change (decrease) in concentrations of the aquatic HSs was observed over all the photolyzing process. However, for the nanotubular-TiO₂ alone, it took about an hour to reduce the concentrations of the aquatic HSs by half; after that period of the photolyzation, significant changes of the HS concentrations were not observed.

The synergistic effect of MWCNTs on the photodegradation of the aquatic HSs was found in an order of 20 > 30 > 10 > 5% of the carbon nanotubes in the MWCNT/nanotubular-TiO₂ composites. Namely, the MWCNT/nanotubular-TiO₂ composites with a weight ratio of 20% MWCNTs provided the best synergistic effect on the photodegradation of the aquatic HS.

The diffuse reflectance UV-vis spectra of the MWCNT/nanotubular-TiO₂ composites (Figure 3) gave new insights into the photodegradation mechanism of the MWCNT/nanotubular-TiO₂ composite catalysts. The nanotubular-TiO₂ has showed the characteristic spectrum of anatase with its fundamental absorption edge rising at 400 nm. The MWCNT/nanotubular-TiO₂ composites, on the other hand, have shifted to shorter wavelength. In other words, combining MWCNT with the nanotubular-TiO₂ has resulted a blue shift. It is noticeable that the MWCNT/nanotubular-TiO₂ composites of 20% MWCNTs provided a largest change of the UV-vis spectrum, followed by 30, 10, and then 5%.

MWCNTs are capable of absorbing the irradiation (photons); this results in production of photoinduced electrons over the carbon nanotubes. The photoinduced electrons transferred from MWCNTs into conduction band of the nanotubular-TiO₂. Superoxide radicals (very reactive) formed over the nanotubu-

lar-TiO₂ surfaces by transferring the photoinduced electrons into the adsorbed oxygen. Highly reactive hydroxyl radicals might be also formed; in case of formation of positively charged holes by electron migration from the nanotubular-TiO₂ valence band to the carbon nanotubes.¹⁸⁻²⁰ These resultant radicals are responsible considerably for the degradation of the aquatic humic substances.

Finally, note here that the thermal stability of the MWCNT/nanotubular-TiO₂ composites were investigated using the thermogravimetric method. TG curve indicated mass loss up to 510 °C of a total 21% for the composites containing 20% MWCNTs.

In conclusion, a new type of photocatalysts was established by combination of the TiO₂-derived nanotubes and the highly-dispersed MWCNTs. The nanotubular shapes of TiO₂ provide the desirable morphologies for attaching the photocatalyst onto the sidewalls of the carbon nanotubes. Identification of the main products by analysis of the photolyzed solutions using LC-MS is under investigation.

References

- 1 A. P. Back, R. F. Christman, *J. Am. Water Works Assoc.* **1963**, 55, 753.
- 2 L. E. Bennett, M. Drikas, *Water Res.* **1993**, 27, 1209.
- 3 M. Hiraide, Y. Arima, A. Mizuike, *Anal. Chim. Acta* **1987**, 200, 171.
- 4 J. F. McCarthy, in *Aquatic Humic Substances: Aquatic Humic Substances: Influence on Fate and Treatment of Pollutants*, ed. by I. H. Suffet and P. MacCarthy, American Chemical Society, Washington, DC, **1989**, p. 263.
- 5 J. J. Rook, *Water Treat. Exam.* **1974**, 23, 234.
- 6 J. J. Rook, *Environ. Sci. Technol.* **1977**, 11, 478.
- 7 W. Aui, S. Tamura, M. Abe, K. Ogino, *Sci. Total Environ.* **1992**, 117-118, 543.
- 8 C. Jucker, M. M. Clark, *J. Membr. Sci.* **1994**, 97, 37.
- 9 J. Zhou, C. J. Banks, *Chemosphere* **1993**, 27, 607.
- 10 P. Backlund, *Chemosphere* **1992**, 25, 1869.
- 11 R. B. Eggins, F. L. Plalmer, J. A. Byrne, *Water Res.* **1997**, 31, 1223.
- 12 X. Liu, J. Yang, L. Wang, X. Yang, L. Lu, X. Wang, *Mater. Sci. Eng. A* **2000**, 289, 241.
- 13 G. Colón, M. C. Hidalgo, J. A. Navío, *Catal. Today* **2002**, 76, 91.
- 14 B. Fugetsu, W. Han, N. Endo, Y. Kamiya, T. Okuhara, *Chem. Lett.* **2005**, 34, 1218.
- 15 T. Kasuga, M. Hiramatsu, A. Hoson, T. Sekino, K. Niihara, *Langmuir* **1998**, 14, 3160.
- 16 T. Kasuga, M. Hiramatsu, A. Hoson, T. Sekino, K. Niihara, *Adv. Mater.* **1999**, 11, 1307.
- 17 C. S. Uyguner, M. Bekbolet, *Desalination* **2005**, 176, 167.
- 18 W. Feng, Y. Feng, Z. Wu, A. Fujii, M. Ozaki, K. Yoshino, *J. Phys.: Condens. Matter* **2005**, 17, 4361.
- 19 W. Wang, P. Serp, P. Kalck, J. L. Faria, *J. Mol. Catal. A: Chem.* **2005**, 235, 194.
- 20 Y. Yu, J. Yu, J. Yu, Y. Kwok, Y. Che, J. Zhao, L. Ding, W. Ge, P. Wong, *Appl. Catal., A* **2005**, 289, 186.

An Ecological Study on the Association of Public Dental Health Activities and Sociodemographic Characteristics with Caries Prevalence in Japanese 3-Year-Old Children

J. Aida^a Y. Ando^b H. Aoyama^c T. Tango^b M. Morita^a

^aDepartment of Preventive Dentistry, Division of Oral Health Science, Hokkaido University Graduate School of Dental Medicine, Sapporo, ^bNational Institute of Public Health, Wako, and ^cTochigi Prefectural Medical and Social Welfare College, Utsunomiya, Japan

Key Words

Ecological study · Empirical Bayes estimation · Fluoride application program

Abstract

The aim of this ecological study was to determine the association of the frequency of dental health activities conducted as public health service and sociodemographic characteristics with caries prevalence in Japanese 3-year-old children using data for each municipality as one unit. Data on caries prevalence in 3-year-old children in 2000 were obtained from 3,251 municipalities (almost all municipalities in Japan). Caries prevalence for each municipality was recalculated using the empirical Bayes estimation model to make adjustments for variations in municipalities with small numbers of children. Explanatory factors included annual frequency of dental health activities such as dental health education and topical fluoride application programs and various sociodemographic characteristics. Multiple linear regression analysis was applied to examine the associations between caries prevalence and annual frequency of dental health activities with adjustment of other sociodemographic variables. Variations in caries prevalence in municipalities with small populations became smaller after being adjusted by the empirical Bayes estimation model. Multiple linear re-

gression analysis showed significant associations of caries prevalence with sociodemographic characteristics such as the proportion of residents with university degrees, total fertility rate, and the proportion of workers in the manufacturing industry ($p < 0.001$). Annual frequency of topical fluoride application showed a significant but relatively weak association with caries prevalence ($p < 0.001$), explaining only 0.8% of the total variation in caries prevalence, whereas no correlation was found between caries prevalence and annual frequency of dental health education. It was concluded that the effect of the fluoride application service on caries prevalence in 3-year-old children, although statistically significant, was of far lower clinical significance than sociodemographic characteristics.

Copyright © 2006 S. Karger AG, Basel

In the last three decades, caries prevalence has been declining in most industrialized countries [Marthaler, 2004]. The decline in caries prevalence in Japanese 3-year-old children started in the 1960–1970s. National surveys have shown that the percentage of caries-free Japanese 3-year-old children increased from 13% in 1969 to 64% in 1999 [Dental Health Division of Health Policy Bureau, Ministry of Health, Labour and Welfare, Japan, 2001].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2006 S. Karger AG, Basel
0008–6568/06/0406–0466\$23.50/0

Accessible online at:
www.karger.com/cre

Manabu Morita
Department of Preventive Dentistry, Division of Oral Health Science
Hokkaido University Graduate School of Dental Medicine
Kita-13, Nishi-7, Kita-ku, Sapporo 060-8586 (Japan)
Tel. +81 11 706 425, Fax +81 11 706 4918, E-Mail mmorita@den.hokudai.ac.jp

Community water fluoridation has not been established in Japan, but topical fluoride application programs and dental health education for parents have been carried out occasionally as part of public dental health services in all Japanese municipalities. Dental health education focuses on plaque control procedures and dietary habits. The criteria for deciding whether to provide a topical fluoride application program and dental health education differ in municipalities. Most of these activities are carried out at a public health center.

An ecological study is one of the procedures for evaluating possible effects on geographic variation. Relationships of caries experience to various health services such as health education, water fluoridation and promotion of the use of fluoride toothpaste have been investigated in ecological studies in western countries [Nadanovsky and Sheiham, 1995; Riley et al., 1999; Blair et al., 2004]. In addition, significant associations have been reported between caries prevalence and social background indices such as ratio of dentists to population, percentage of women in the labor force, level of education [Amstutz and Rozier, 1995; Nadanovsky and Sheiham, 1995; Riley et al., 1999; Thomson and Mackay, 2004; Truin et al., 2005]. However, there has been no nationwide study on the effects of these public dental health activities and sociodemographic characteristics on caries experience in Japan. In Japan, dental examinations are conducted for 3-year-old children in all municipalities every year. More than 90% of children have participated in the examinations. Most of these records have recently been stored in a community oral health databank [8020 Promotion Foundation, 2004]. Nationwide data are therefore now available for an ecological study.

However, caries prevalence in municipalities varies depending on the population, especially for rural areas in which small numbers of children have been examined. An ecological approach therefore lacks precision due to the large variation in caries prevalence resulting from sampling error. In order to increase the precision, an empirical Bayes estimation model has been employed [Elliott et al., 1997; Tango, 1999]. This method logically corrects the disease prevalence by taking into account the variation in sample size [Benach et al., 2003; Yiannakoulas et al., 2003; Yu et al., 2004].

The aim of this ecological study was to determine the relationships of caries prevalence estimated using the empirical Bayes approach with frequency of public dental health activities and sociodemographic characteristics among Japanese 3-year-old children.

Materials and Methods

Study Sample

The present study is an ecological study in which community-based variables were used. There are 3,252 municipalities in the 48 prefectures of Japan. The caries prevalences (percentage of 3-year-old children with caries) from all municipalities have been stored in the Community Oral Health Databank [8020 Promotion Foundation, 2004]. Since we targeted all of the municipalities in Japan, no sampling methods were used.

Dental Health-Related Factors

The factors related to dental health were obtained from various databases. The annual frequencies of dental health education per child and topical fluoride application program per child, conducted at regional health centers, were obtained from the Report on Public Health Center Activities and Health Services [Statistics and Information Department of Minister's Secretariat, Ministry of Health, Labour and Welfare, 2000a]. Dichotomous variables such as the presence or absence of dentists or dental hygienists at community health facilities were obtained from the same reports. Data on the ratio of dentists working at private dental offices in each municipality (dentists:population ratio) were obtained from the national report on health facilities investigation [Statistics and Information Department of Minister's Secretariat, Ministry of Health, Labour and Welfare, 2002b]. The percentage of 3-year-old children in each municipality who underwent dental health examinations was also calculated. It is possible that dental health-related factors, including the frequency of preventive measures in private dental clinics, availability of fluoride toothpaste, sugar consumption, food habits and oral hygiene, affect caries prevalence. However, no information was available.

Sociodemographic Characteristics

Sociodemographic information for each municipality, including population, total fertility rate, unemployment rate, industrial structure, family structure and educational level, was obtained from the Population Census and Vital Statistics of Japan [Statistics Bureau, Ministry of Internal Affairs and Communications, 2002; Statistics and Information Department of Minister's Secretariat, Ministry of Health, Labour and Welfare, 2002c]. The industrial structure of a community was expressed as industrial structure 1 (primary industries such as farming and fisheries), industrial structure 2 (secondary industries such as manufacturing) and industrial structure 3 (tertiary industries such as services and commerce). The percentage of workers in category 1 in a municipality was expressed as industrial structure 1 rate. Data on family structure included the average size of a family, nuclear family rate, proportion of households with a person of 65 years or older, and the percentage of 3-year-old children attending nursery schools. The proportions of residents in a municipality with certain levels of education, i.e., junior high school, senior high school, junior college and university, were used to express the educational level of a municipality. Data on average income of individuals were obtained from the report on individual income indices [Japan Marketing Education Center, 1999]. Data on expenditure for public health activities per child in each municipality were also obtained.

Empirical Bayes Estimation

In an area with a small population, dental caries prevalence is greatly affected by population size. Therefore, empirical Bayes estimation of caries prevalence was calculated to provide more stable estimates (d = number of children with at least one dmf tooth or more; θ = percentage of children with at least one dmf tooth or more (prevalence); n = total number of children examined).

The probability density function of d is based on a binomial distribution:

$$f(d|\theta, n) = {}_n C_d (\theta)^d (1 - \theta)^{n-d}.$$

According to the Bayes' theorem, the probability density function of prior distribution of θ is based on a β distribution:

$$p(\theta|\alpha, \beta) = \theta^{\alpha-1} (1 - \theta)^{\beta-1} / B(\alpha, \beta),$$

$B(\alpha, \beta)$ being based on a β distribution.

The empirical Bayes estimation of dental caries prevalence is given below.

$$\text{Expectation value: } E(\hat{\theta}) = (\alpha + d) / (\alpha + \beta + n),$$

$$\text{Variance: } \text{Var}(\hat{\theta}) = (\alpha + d)(\beta + n - d) / (\alpha + \beta + n)^2 (\alpha + \beta + n + 1).$$

Statistics

Simple linear regression analysis was used to assess the correlations of all explanatory variables with caries prevalence. At first, non-normality in the distribution of variables was corrected. Square root transformation was applied to the annual frequency of dental health education, annual frequency of topical fluoride application, dentist:population ratio, and rate of industrial structure 1 in each municipality. Natural log transformation was applied in the case of population, proportion of participants in dental health examination, and proportion of residents having graduated from universities.

Multiple linear regression analysis was used to adjust the independent effects of dental health service indices on prevalence of dental caries. Explanatory variables included dental health-related community data and sociological data. The level of significance was set at 5%. In order to prevent multicollinearity in the regression model, some variables that were strongly correlated with other variables ($r < -0.8$ or $r > 0.8$) were deleted. Then independent variables were used for multiple linear regression analysis.

Based on the results of multiple linear regression analysis, a figure of residual plots was obtained. The purpose of the residual plots was to demonstrate the relationships between caries prevalence and public dental health service indices (annual frequency of topical fluoride application or dental health education) after adjusting other factors. In other words, the residual plot shows the effect of topical application of fluoride or dental health education on caries prevalence without effects of other explanatory variables.

Empirical Bayes estimation of caries prevalence was calculated using EB estimation for Binomial-Beta software [Takahashi, 2004]. Statistical analysis was performed using SPSS version 11.5 and STATA version 7.0 software.

Results

Caries data were obtained from almost all of the municipalities in Japan (3,251 out of 3,252 municipalities). Figure 1 shows a scatter plot indicating the association

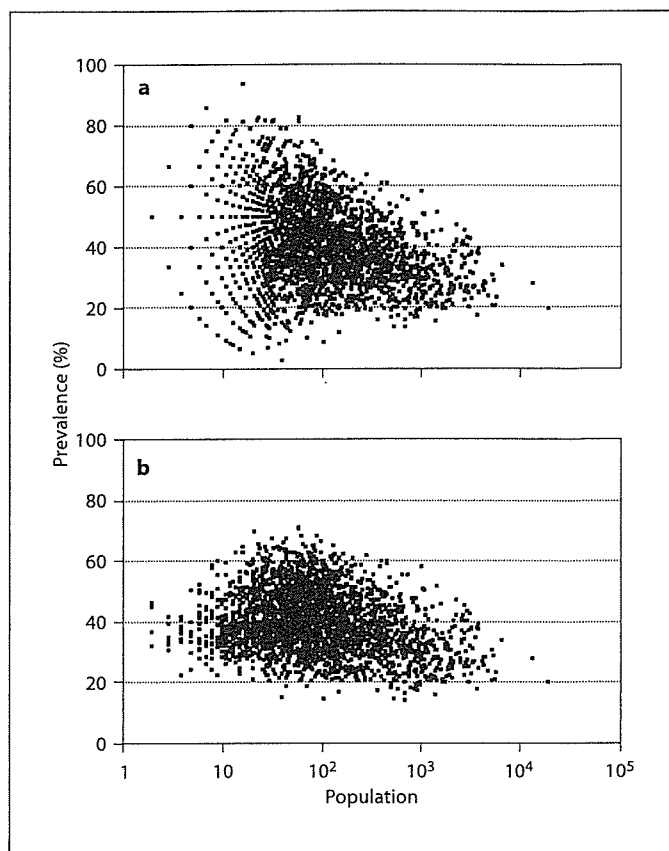


Fig. 1. Scatter plot of caries prevalence by number of children examined in each municipality (logarithmic scale). Crude caries prevalence (a) and empirical Bayes estimate of caries prevalence (b).

between caries prevalence and number of examined children before and after the application of an empirical Bayes estimation model. Logarithmic scale was applied to the x axis (population). Before application of the empirical Bayes estimation model, caries prevalence varied depending on the number of children examined (fig. 1a). Mean (\pm standard deviation) caries prevalence was $40.4 \pm 14.2\%$, and caries prevalence ranged from 0 to 100%. Variation among municipalities became smaller after the application of empirical Bayes estimation, especially among areas with small numbers of children, indicating a decrease in sampling error (fig. 1b). After application of empirical Bayes estimations, mean caries prevalence was $39.8 \pm 10.0\%$ and caries prevalence ranged from 14.3 to 71.1%. Figure 2 shows a map of caries prevalence in Japan with marked geographic differences in prevalence.

The results of simple linear regression analysis (table 1) showed that caries prevalence had a statistically sig-

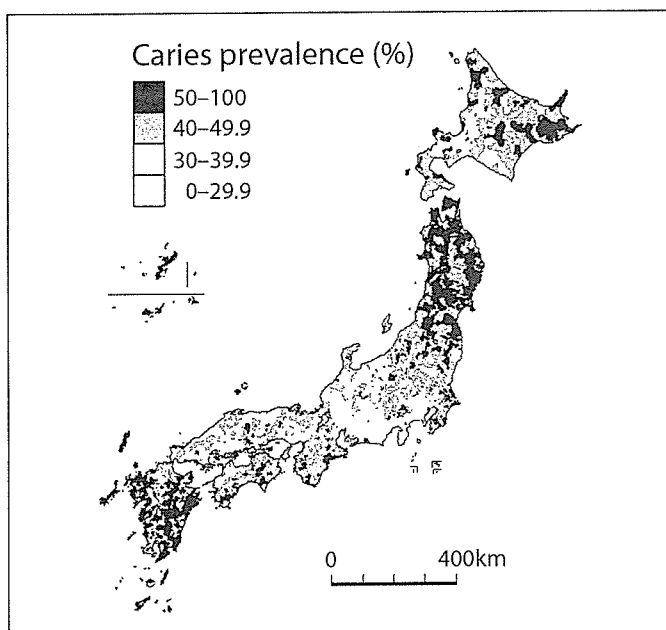


Fig. 2. Caries map of Japanese 3-year-old children.

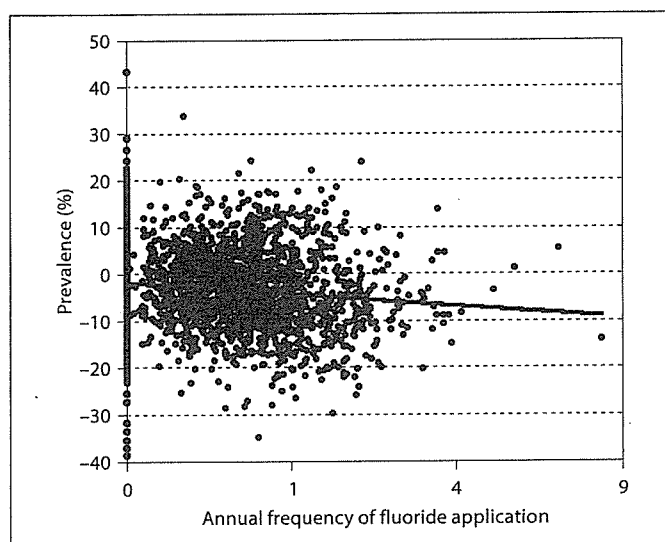


Fig. 3. Residual plot of caries prevalence in each municipality demonstrating relationships between caries prevalence and frequency of topical fluoride application after adjusting sociodemographic indices.

Table 1. Mean and standard deviation (SD) values of explanatory variables and their Pearson's correlation coefficients (r) with caries prevalence adjusted using the empirical Bayes estimation model

Explanatory variables	Mean	SD	r	p value
Frequency of topical fluoride application per year	0.348	0.600	-0.075	<0.001
Frequency of dental health education per year	0.892	0.799	-0.030 ^c	0.083
Dentist employed at the health center ^a	0.024	0.141	-0.039	0.027
Dental hygienist employed at the health center ^a	0.094	0.284	-0.161	<0.001
Dentist:population ratio	0.0005	0.0009	-0.150 ^c	<0.001
Ratio of participants in dental examinations	0.905	0.090	0.054 ^d	0.002
Population	38,951	86,991	-0.286 ^d	<0.001
Unemployment rate	0.308	0.013	0.081	<0.001
Industrial structure 1 (farming, fishery) rate ^b	0.140	0.106	0.420 ^c	<0.001
Industrial structure 2 (manufacturing) rate	0.308	0.086	-0.138	<0.001
Industrial structure 3 (services, commerce) rate ^b	0.511	0.096	-0.284	<0.001
Total fertility rate	1.543	0.202	0.387	<0.001
Size of family	3.031	0.464	0.125	<0.001
Nuclear family rate	0.545	0.084	-0.190	<0.001
Proportion of households with elderly 65 years or older ^b	0.507	0.135	0.267	<0.001
Proportion of children attending nursery school	0.420	0.179	0.178	<0.001
Proportion of junior high school graduates ^b	0.301	0.105	0.414	<0.001
Proportion of senior high school graduates	0.352	0.050	-0.150	<0.001
Proportion of junior college graduates ^b	0.070	0.021	-0.514	<0.001
Proportion of university graduates	0.065	0.037	-0.507 ^d	<0.001
Average income, USD ^b	31,590	445.1	-0.491	<0.001
Rate of expenditure for public health, USD	30.7	3.1	0.201	0.001

^a 0 = Not employed; 1 = employed.

^b Variables which were not included in the multiple regression analysis.

^c Pearson's correlation coefficient (r) was calculated after square root transformation.

^d Pearson's correlation coefficient (r) was calculated after log transformations.

Table 2. Stepwise multiple linear regression of caries prevalence (%) adjusted by empirical Bayes estimation

	Correlation coefficient (95% CI)	Standardized regression coefficient	Partial correlation coefficient	p value	Explained variation %
Frequency of topical fluoride application ¹	-2.5 (-3.2 to -1.7)	-0.103	-0.116	<0.001	0.797
Frequency of dental health education ¹	-0.6 (-1.5 to 0.4)	-0.018	-0.020	0.262	0.058
Dentist employed at the health center	-0.5 (-2.6 to 1.5)	-0.008	-0.009	0.608	0.030
Dental hygienist employed at the health center	-0.6 (-1.8 to -0.5)	-0.016	-0.019	0.288	0.266
Dentist:population ratio ¹	-3.9 (-43.6 to 35.7)	0.003	0.003	0.846	0.044
Ratio of participants in dental examinations ²	3.4 (0.7 to 6.1)	0.036	0.044	0.012	0.189
Industrial structure 2 (manufacturing) rate	-24.8 (-28.7 to -20.9)	-0.193	-0.214	<0.001	2.651
Unemployment rate	119.9 (98.4 to 141.5)	0.172	0.190	<0.001	1.326
Total fertility rate	10.0 (8.4 to 11.6)	0.198	0.216	<0.001	7.657
Number in a household	1.4 (0.6 to 2.2)	0.059	0.062	<0.001	0.742
Proportion of children attending nursery school	-2.9 (-4.8 to -1.1)	-0.048	-0.055	0.002	-0.863
Proportion of university graduates ²	-10.3 (-11.1 to -9.5)	-0.485	-0.414	<0.001	25.996
Rate of expenditure for public health	0.4 (-0.1 to 0.9)	0.025	0.152	0.152	0.495
Total					39.4

R² value = 0.394.
¹ Square root transformation.
² log transformations.

nificant association ($p < 0.001$) with some explanatory variables. Annual frequency of topical fluoride application showed a very low correlation coefficient ($r = -0.075$) compared with the other sociodemographic factors such as proportion of residents with university degrees ($r = -0.507$), average income ($r = -0.491$), and total fertility rate ($r = 0.387$).

Industrial structure 1 rate, industrial structure 3 rate, proportion of junior high school graduates, proportion of junior college graduates, and average income (USD) showed significant correlations with proportion of university graduates ($r < -0.8$ or $r > 0.8$). Rate of households with persons 65 years or older was significantly correlated with nuclear family rate ($r < -0.8$). These six variables were deleted from the multiple linear regression analysis (table 1).

The results of backward stepwise multiple linear regression analysis (table 2) showed that caries prevalence in each municipality was significantly associated with annual frequency of topical fluoride application (partial correlation coefficient = -0.116 , $p < 0.001$). The frequency of dental health education (partial correlation coefficient = -0.020) was not significantly related to caries prevalence ($p = 0.262$). Sociodemographic indices such as the proportion of university graduates and the propor-

tion of industrial structure 2 (manufacturing) were significantly associated with caries prevalence (partial correlation coefficients = -0.414 and -0.214 , respectively; $p < 0.001$) followed by total fertility rate (partial correlation coefficient = 0.216 , $p < 0.001$). The term 'percentage of explained variation' indicates the percentage of variation explained by each variable in total variation of caries prevalence. The proportion of university graduates and total fertility rate explained 26.0 and 7.7%, respectively. On the other hand, the frequency of topical fluoride application and the frequency of dental health education explained little (0.8 and 0.06%, respectively). About 39% (R² values) of the variance in the caries prevalence could be accounted for by explanatory variables.

Residual plot of caries prevalence in each municipality obtained from multiple linear regression analysis is shown in figure 3. It demonstrates the relationship between caries prevalence and annual frequency of topical fluoride application after adjusting for other explanatory variables. The x axis (annual frequency of topical fluoride application) is in square scale because square transformation had been applied in the multiple linear regression. The frequency of topical fluoride application after adjusting for sociodemographic indices showed a very weak negative correlation with caries prevalence.

Discussion

Although significant factors related to caries prevalence were elucidated in the present study, there are some limitations. First, this study was a cross-sectional study, not a cohort study, and causal relationship could therefore not be clarified. Second, data that affect caries prevalence more directly, such as fluoride dentifrice consumption and sugar consumption, were not used. Additionally, aggregated data in municipalities, not individual data, were used. It is possible that municipality data do not always reflect an individual level.

Variations in small areas have been a problem in epidemiological research [Elliott et al., 1997]. When the empirical Bayes estimation was applied, the variation in caries prevalence, especially that in areas with small numbers of children, decreased (fig. 1). This finding indicates the usefulness of the empirical Bayes estimation model as shown in previous medical studies [Benach et al., 2003; Yiannakoulis et al., 2003; Yu et al., 2004]. For example, Yiannakoulis et al. [2003] used the model for estimating injury incidence and Yu et al. [2004] used the model for estimating cancer survival. In dental studies, Antunes et al. [2002] used this method for estimation of caries risk. Leroux et al. [1996] used mixed-effect regression models for estimating caries prevalence in small areas. Both of these methods have a distinct advantage over other traditional methods for estimations in small areas [Ghosh and Rao, 1994].

Multiple linear regression analysis demonstrated statistically significant ecological relationships between caries prevalence and annual frequency of topical fluoride application. However, the relationship was weak compared to the sociodemographic characteristics. The percentage of variation explained by topical fluoride application in total variation of caries was 0.8%. On the other hand, the effect of topical fluoride application reported by the Cochrane review was substantial [Marinho et al., 2002]. This discrepancy might have resulted from our study limitations stated above. The Cochrane review was based on data for individuals who received topical fluoride application. The effect of topical fluoride application as public dental health service has been shown to be statistically significant, though the clinical effect is less than the effect of sociodemographic characteristics.

Another reason for the small clinical effect of fluoride application in this study is 'temporal relationships'. For example, it is possible that high caries risk children received topical fluoride application preferentially in some municipalities. As mentioned previously, this is a cross-

sectional study. While two factors occur together, this does not imply that the presence of one leads to the other. Therefore, the results might show temporal relationships of the association. In order to judge whether the relation between two factors is causal or just an association, commonly used criteria (Bradford Hill's criteria) might be applicable [Daly et al., 2003]. However, we could not confirm this relation in this ecological study.

Our cross-sectional ecological study also showed that dental health education had little effect on caries prevalence. Previous intervention studies have yielded diverse results. Vanobbergen et al. [2004] carried out an oral health education program for primary school children. The program was shown to be effective in improving reported dietary habits and the proper use of topical fluorides, resulting in a higher dental care index. However, there was no significant reduction in caries prevalence. Blair et al. [2004] evaluated oral health promotion programs in an ecological study. They carried out multiple intervention programs in most socioeconomically deprived communities to reduce inequalities in preschool children. Reductions in mean dmft of 46% for 36- to 47-month-old children and 37% for 48- to 59-month-old children were found in the pilot public health program area over a 4-year period, whereas increases in mean dmft for 36- to 47-month-old and 48- to 59-month-old children were found in the control area. These results suggest the need for a more effective community dental health education program.

Our study showed relatively strong associations between sociodemographic indices and community-based caries prevalence. Results of many previous studies, including ecological studies, support our results [Amstutz and Rozier, 1995; Ndanovsky and Sheiham, 1995; Riley et al., 1999; Thomson and Mackay, 2004; Truin et al., 2005]. Ndanovsky and Sheiham [1995] carried out multiple linear regressions including caries reduction, ratio of dentists to population and social background variables in an international ecological study. They reported that dental service explained only 3% of the variation in caries level of 12-year-olds. However, broad social background variables such as the percentage of women in the labor force, life expectancy, and level of education explained 65% of the variation.

Regional disparities on caries prevalence in Japanese 3-year-old children were shown (fig. 2). It has been reported that preventive methods such as water fluoridation or salt fluoridation reduce the disparities of caries prevalence affected by socioeconomic background [Riley et al., 1999; Watt and Sheiham, 1999; McDonagh et al.,