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Effective UV/Ozone irradiation method for decontamination of hydroxyapatite surfaces

Keisuke Yasuda, Yohei Okazaki, Yasuhiko Abe *, Kazuhiro Tsuga

Department of Advanced Prosthodontics, Division of Dental Sciences, Biomedical Sciences Major, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

* Corresponding author at: Department of Advanced Prosthodontics, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734–8553, Japan.

E-mail address: abey@hiroshima-u.ac.jp (Y. Abe).

Abstract

The purpose of this study was to establish whether UV/ozone (O_3) irradiation method can effectively decontaminate hydroxyapatite surfaces, including those modified by the treatment with 30% phosphoric acid solution through morphological and chemical surface analyses (surface roughness, X-ray photo-electron spectroscopy and wettability), and to evaluate the *in vitro* response of osteoblast-like MC3T3-E1 cells to the modified hydroxyapatite surface decontaminated via this method. The amount of carbon and the contact angle of hydroxyapatite surfaces were significantly decreased by UV/O_3 irradiation that lasted for ≥ 5 and ≥ 3 min, respectively ($P < 0.01$). Additionally, 7-day storage of H_3PO_4-modified hydroxyapatite surface decontaminated with 5-min irradiation did not affect contact angle values ($P > 0.05$). MC3T3-E1 cell proliferation, differentiation (as assessed by relative *ALP* and *OCN* mRNA levels), and mineralisation were significantly promoted on irradiated surfaces ($P < 0.05$). These findings show that UV/O_3 irradiation for ≥ 5 min significantly decontaminated H_3PO_4-modified hydroxyapatite surface, improved its wettability, and facilitated osteoblast growth and function.
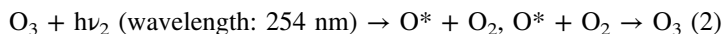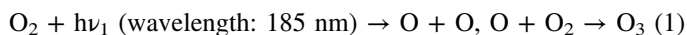
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1. Introduction

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HA) is a candidate bioactive material for bone tissue engineering scaffolds along with β-tricalcium phosphate [$β$-$Ca_3(PO_4)_2$] [1, 2]. HA is highly thermodynamically stable at a physiological pH (7.4) but is not bioresorbable [3].

The integration of HA surface with live tissue involves multiple steps including HA solubilisation, establishment of the equilibrium between HA surface and aqueous solutions, protein adsorption, and cell adhesion. According to Dorozhkin [4, 5], HA dissolution occurs in five steps, during which the composition of the surface changes to tricalcium phosphate [$Ca_3(PO_4)_2$] and dicalcium phosphate dehydrate [$CaHPO_4$] (DCPD). Bertazzo et al. [6] proposed three initial phases in the five steps that occur before the equilibrium is reached between the modified HA surface and biological fluids, with the formation of DCPD on this surface. In our previous study [7], we verified that etching with 30% phosphoric acid of the nanostructured HA surface effectively altered the surface Ca/P ratio and accelerated the initial adhesion, proliferation, and differentiation of osteoblast-like MC3T3-E1 cells. Additionally, bioactive HA surface also enhanced the adhesion and differentiation of RAW 264.7 leukemic macrophage cells, as well as demonstrated favourable properties of a superior surface for bone tissue engineering [8].

The ultraviolet/ozone (UV/O_3) irradiation method has been generally used for removal of contaminants from the surfaces of ceramic materials [9]. In that approach, decontamination of ceramic materials occurs via the action of O_3 and excited oxygen (O*) atom from atmospheric oxygen (O_2) formed by short wavelength UV light irradiation (UV-C: 185 nm and 254 nm). O_3 and O* are produced by the following reaction [9]:

$O_2 + hν_1$ (wavelength: 185 nm) $→ O + O, O + O_2 → O_3$ (1)

$O_3 + hν_2$ (wavelength: 254 nm) $→ O* + O_2, O* + O_2 → O_3$ (2)

After Wang et al. demonstrated that UV irradiation induced amphiphilic surfaces of titanium dioxide [10], the UV/O_3 irradiation method has been applied to biomaterials such as titanium [11] and zirconium [12]. However, the application of UV/O_3 irradiation for the decontamination of HA as a biomaterial has not been reported.

Therefore, the purpose of this study was to examine whether UV/O_3 irradiation effectively decontaminates HA surfaces modified by treatment with 30% phosphoric acid solution [7], by morphological and chemical surface analyses. We also evaluated the *in vitro* response of osteoblast-like cells to the modified HA surface decontaminated with this method.

2. Materials and methods

2.1. Validation of the UV/O$_3$ irradiation method for untreated HA surfaces and surfaces modified by 30% phosphoric acid solution

2.1.1. Sample preparation

HA plates (thickness, 2 mm; width, 10 mm; length, 10 mm) (APP-100; Pentax, Tokyo, Japan) were used in this study. HA plates were either untreated or exposed to 30% phosphoric acid [H$_3$PO$_4$] (lot no. T1949; Sigma-Aldrich Japan, Tokyo, Japan) solution for 10 min at 25 °C, followed by rinsing three times with ultrapure water (MilliQ water: >18 MΩcm) (HAP). The untreated and treated HA samples were irradiated with UV/O$_3$ for 1, 2, 3, 4, 5, 10, 30, 60, or 120 min using a UV/O$_3$ cleaner (ASM401; Asumi, Tokyo, Japan). The apparatus contained low-pressure mercury UV-light that emitted UV-light at 185-nm (1.5 mW/cm^2) and 254-nm (15 mW/cm^2) wavelengths [13]. The energy of UV-emissions was 647 KJ/mol for 185 nm and 472 KJ/mol for 254 nm. The distance between the UV-source and the sample was 20 mm. Non-irradiated samples were used as controls. Additionally, a modified HA sample irradiated with UV/O$_3$ for 5 min was used to verify the preservation of contact angle during 7 day-storage at room temperature. The surface characteristics of the irradiated samples and controls were evaluated by the following methods. For all samples, each evaluation was conducted in triplicate.

2.1.2. Morphological analysis

Untreated samples and those treated with 30% H$_3$PO$_4$ were exposed to UV/O$_3$ irradiation or kept intact. They were observed for 120 min using a scanning electron microscope (SEM) (VE-8800, Keyence, Japan) and the surface roughness (Ra) was measured using a confocal scanning laser microscope (VK-8500, Keyence, Japan). The Ra value (μm) was defined as the average value of five 100 μm × 100 μm areas.

2.1.3. X-ray photoelectron spectroscopy (XPS) analysis

The samples that had been exposed to UV/O$_3$ irradiation or left intact were mounted individually onto stubs with insulating tape. The surfaces of the samples were chemically analysed using an XPS instrument (AXIS-HS; Kratos, Manchester, UK). The measurements were performed *in vacuo* ($\leq 10^{-7}$ Pa) with Al-Kα monochromatic X-rays at a source power of 150 W. The AXIS-HS instrument was equipped with an electron flood gun for charge compensation. Wide- and narrow-scan spectra were acquired at pass energies of 80 eV and 40 eV, respectively. Peak positions were calibrated by referencing a value of 284.6 eV for the peaks corresponding to C-C and C-H in the C 1s spectrum. After smoothing the

narrow scans, a straight line (for C 1s, O 1s, Ca 2p, and P 2p) was applied in quantification. The relative sensitivity factors used to calculate the atomic ratios from the peak area ratios were 0.278 for C 1s, 0.780 for O 1s, 1.833 for Ca 2p, and 0.486 for P 2p. Reproducibility was ensured by obtaining five measurements per experimental sample.

2.1.4. Wettability

The wettability of sample surfaces was measured using a contact angle meter (Simage mini; Excimer, Kanagawa, Japan) with the sessile drop method and 3.6 μL ultrapure water. The Young's equation was used for data analysis.

2.2. Osteoblast-like cells response to the modified HA surface decontaminated by UV/O_3 irradiation

2.2.1. Sample preparation

Untreated samples and samples treated with 30% H_3PO_4 were irradiated with UV/O_3 for 5 min or left intact. Their characteristics were evaluated by the following methods. For all samples, each evaluation was conducted in triplicate.

2.2.2. Proliferation of osteoblast-like cells

MC3T3-E1 osteoblast-like cells (derived from mouse calvaria (RIKEN BioResource Center, Tsukuba, Japan)) were cultured in Dulbecco's modified Eagle's medium (D-MEM) (lot no. RNBD8499; Sigma-Aldrich Japan) containing 10% foetal bovine serum (lot no. 1324098; Sigma-Aldrich Japan) and 1% penicillin-streptomycin (Sigma- Aldrich Japan) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed twice a week.

MC3T3-E1 cells (5×10^3) were seeded onto the 5-min-irradiated and control samples. The cells were incubated at 37 °C in the atmosphere of 95% air and 5% CO_2. Cell proliferation was evaluated at 1, 3, and 5 days of culture. Additional cells were seeded on tissue culture plates and incubated as controls over each incubation period.

Cell proliferation was analysed by a Cell Counting Kit-8 assay (Dojindo Laboratory, Kumamoto, Japan), which is a colorimetric test that uses a tetrazolium salt, WST-8, to determine viable cell number. After each incubation period, the growth medium in each well was removed by aspiration and replaced with a mixture of WST-8 and D-MEM in a 1:10 ratio. After 2-h incubation at 37 °C in the atmosphere of 95% air and 5% CO_2, a 110-μL aliquot of the mixture that reacted with viable cells was dispensed into each well (n = 5) of a 96-well microplate. The absorbance was read at 450 nm using a microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, USA).

2.2.3. Differentiation and mineralisation of osteoblast-like cells

MC3T3-E1 cells (10×10^4) were seeded onto the 5-min-irradiated and control samples, as described previously. After a confluent cell monolayer was obtained, minimum essential medium alpha modification (α-MEM: lot no. RNBD3420; Sigma-Aldrich Japan) supplemented with osteoblast-inducer reagent (1% ascorbic acid, 0.2% hydrocortisone, and 2% β-glycerophosphate) replaced D-MEM. Real-time polymerase chain reaction (PCR) analysis of the expression levels of the alkaline phosphatase (*ALP*) and osteocalcin (*OCN*) genes was carried out at 7, 14, and 21 days. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). The total RNA concentration was adjusted to 30 ng/μL using a NanoDrop 1000 system (Thermo Fisher Scientific, Wilmington, DE, USA). One-step PCR was performed from total RNA (30 ng/μL) using a One Step SYBER PrimeScript RT-PCR Kit (TaKaRa, Shiga, Japan) and a Roche LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). Real-time PCR analysis was performed for *ALP*, *OCN*, and *GAPDH* (internal control). The sequences of the primers used in this analysis were as follows: forward, 5′-AGGGTGGACTACCTCTTAGGTCT-3′ and reverse, 5′-TGGTCAATCCTGCCTCCTTCC-3′ for *ALP*; forward, 5′-CACAG-CAGCTTGGCCCAG-3′ and reverse, 5′-CCTGCTTGGACATGAAGGCTTT-3′ for *OCN*; and forward, 5′-GCCAGCCTCGTCCCGTAG-3′ and reverse, 5′-CAAATGGCAGCCCTGGTGAC-3′ for *GAPDH*. The primers were purchased from Sigma.

At 28 days, cell mineralization was evaluated by alizarin red S staining. The growth medium in each well was removed by aspiration and the cells were washed three times with phosphate-buffered saline. After fixation with methanol at 4 °C for 20 min, cells were stained using a calcified nodule staining kit (Cosmo Bio, Tokyo, Japan). Stained samples were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA) as luminance distribution.

2.3. Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) and post hoc Tukey's test for multiple comparisons, if appropriate. Pairwise comparisons were performed by the Student's *t*-test. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Validation of the UV/O$_3$ irradiation method

3.1.1. Morphological analysis

SEM images of the HA samples (untreated or modified by 30% H_3PO_4) that underwent UV/O$_3$ irradiation at 120 min or were kept intact are shown in Fig. 1.
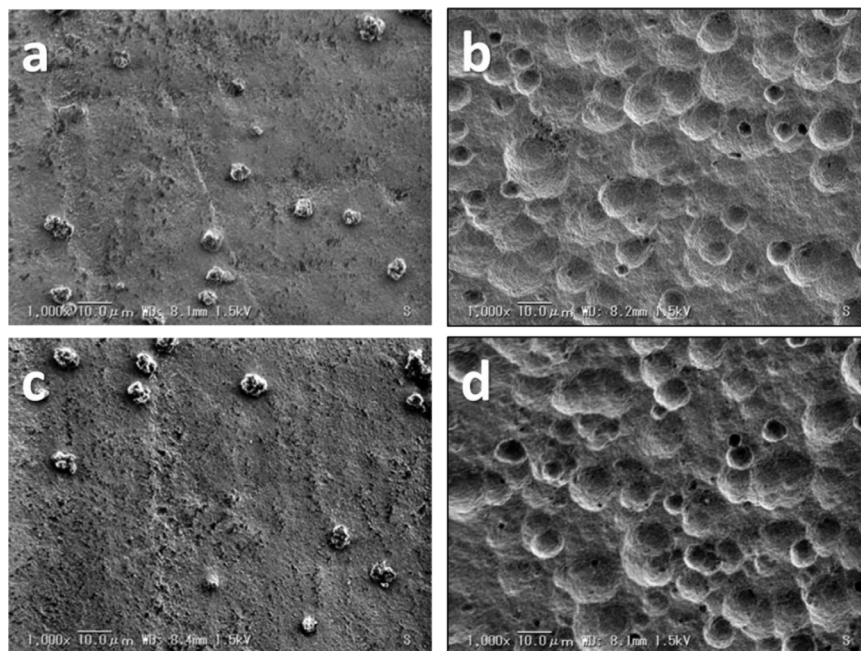
Fig. 1. SEM images of untreated HA surface (a) and H_3PO_4-modified HA surface (b) that did not undergo ultraviolet/ozone (UV/O_3) irradiation, and untreated HA surface (c) and H_3PO_4-modified HA surface (d) exposed to UV/O_3 irradiation for 120 min.

The traces lacking particles appeared at the surface of H_3PO_4-modified samples. The surface roughness (Ra) values of the samples are given in Table 1. Ra values of samples treated with 30% H_3PO_4 were significantly larger than those of untreated samples ($P < 0.01$), whereas UV/O_3 irradiation did not have a significant effect.

3.1.2. XPS analysis

XPS-determined atomic concentrations (at. %) and Ca/P ratios in untreated and H_3PO_4-modified HA samples that were either kept intact (time 0) or exposed to

Table 1. Effect of UV/O_3 irradiation on surface roughness of untreated and H_3PO_4-modified HA samples.

Sample	Irradiation time (min)	Surface roughness Ra (μm)	
Untreated surface	0	0.23 ± 0.02	a
	120	0.21 ± 0.03	a
Modified surface	0	0.91 ± 0.04	a
	120	0.86 ± 0.03	a

[a]$P > 0.05$ indicates no significant difference between non-irradiated samples and samples irradiated for 120 min.

UV/O$_3$ irradiation of various duration are shown in Table 2. For both types of samples, no significant differences in Ca/P ratios were noted between irradiated and control (irradiation time 0) samples ($P > 0.05$). The effect of UV/O$_3$ irradiation on at. % values of carbon (C) in untreated and H$_3$PO$_4$-modified HA samples is shown in Fig. 2. The amount of C in untreated samples decreased with the increase of irradiation time (to less than 10%) after irradiation for over 10 min ($P < 0.01$). The amount of C on H$_3$PO$_4$-modified samples also decreased with the increase of irradiation time, most significantly at $\geq$ 5 min-irradiation time ($P < 0.01$).

3.1.3. Wettability

The effect of UV/O$_3$ irradiation on contact angles of untreated and H$_3$PO$_4$-modified HA samples is shown in Fig. 3. The contact angle of untreated samples at

Table 2. Effect of UV/O$_3$ irradiation duration on XPS-determined atomic concentration (at. %) and Ca/P ratios of untreated and H$_3$PO$_4$-modified HA samples.

| Sample	Irradiation time (min)	At. % O 1s			C 1s			Ca 2p			P 2p			Ca/P			
Untreated surface	0	39.57	±	3.61	30.63	±	2.32	17.37	±	0.98	11.20	±	0.74	1.55	±	0.04	a
	1	48.38	±	1.27	21.21	±	1.80	18.57	±	0.48	11.71	±	0.36	1.59	±	0.05	a
	2	48.94	±	1.50	22.30	±	2.17	17.49	±	0.57	11.27	±	0.36	1.55	±	0.06	a
	3	52.05	±	0.76	19.46	±	1.76	17.37	±	0.58	11.16	±	0.62	1.56	±	0.06	a
	4	54.45	±	0.71	15.68	±	1.25	18.39	±	0.45	11.48	±	0.36	1.60	±	0.05	a
	5	53.17	±	0.57	14.45	±	0.94	19.73	±	0.28	13.54	±	0.47	1.58	±	0.07	a
	10	56.88	±	2.25	8.12	±	0.28	21.46	±	1.66	13.54	±	0.66	1.63	±	0.09	a
	30	55.26	±	0.60	9.19	±	0.75	22.03	±	0.20	13.52	±	0.30	1.63	±	0.03	a
	60	58.28	±	0.72	6.31	±	0.57	21.78	±	0.32	13.65	±	0.29	1.60	±	0.05	a
	120	59.17	±	1.87	5.24	±	0.82	21.86	±	1.11	13.73	±	0.21	1.59	±	0.07	a
Modified surface	0	46.62	±	2.33	24.83	±	0.99	16.62	±	3.90	11.94	±	0.65	1.39	±	0.02	a
	1	50.71	±	1.23	18.59	±	0.29	18.05	±	1.25	12.65	±	0.27	1.43	±	0.03	a
	2	48.93	±	1.27	24.65	±	0.72	15.26	±	2.35	11.16	±	0.40	1.37	±	0.02	a
	3	54.79	±	0.96	12.46	±	0.40	18.96	±	1.57	13.79	±	0.30	1.37	±	0.03	a
	4	53.27	±	0.66	13.51	±	0.43	19.41	±	1.15	13.81	±	0.28	1.41	±	0.04	a
	5	55.83	±	0.53	6.95	±	0.36	21.78	±	0.80	15.46	±	0.57	1.41	±	0.05	a
	10	56.51	±	3.23	6.74	±	0.26	21.80	±	0.75	15.07	±	0.36	1.45	±	0.04	a
	30	56.83	±	0.73	6.93	±	0.44	21.53	±	1.24	14.72	±	0.36	1.46	±	0.04	a
	60	56.81	±	1.02	6.21	±	0.40	21.78	±	1.33	15.20	±	0.25	1.43	±	0.04	a
	120	56.94	±	0.71	6.82	±	0.48	21.67	±	0.96	14.57	±	0.24	1.49	±	0.03	a

[a] $P > 0.05$ indicates no significant difference between samples of untreated and H$_3$PO$_4$ modified surfaces exposed to irradiation of the same duration, respectively.
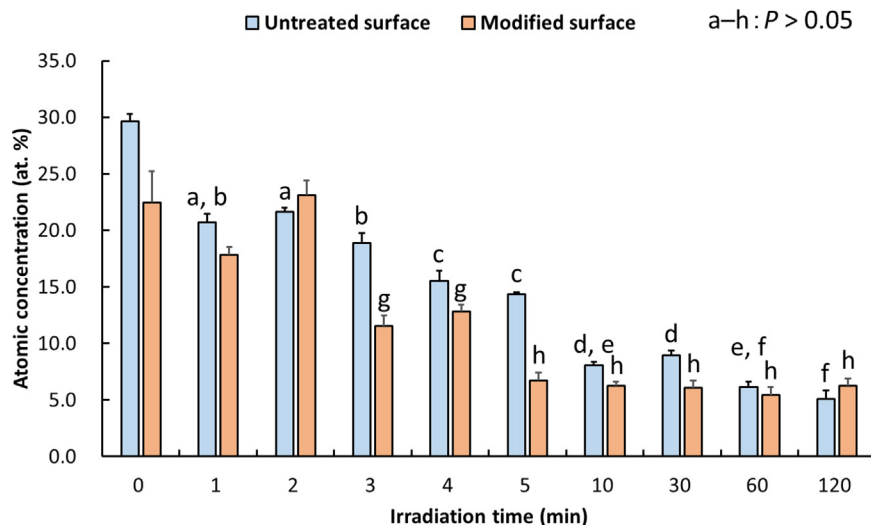
Fig. 2. Mean atomic concentration (at. %) values of carbon (C) in untreated and H_3PO_4-modified HA surfaces irradiated with ultraviolet/ozone (UV/O_3). The same letters (a to h) above the values indicate that there were no significant differences between the two types of samples ($P > 0.05$). The error bars indicate standard deviation.

0 min-irradiation time was > 100 degrees (hydrophobic surface). The contact angle decreased with the increase of irradiation time, most significantly at $\geq$ 5 min-irradiation time ($P < 0.01$) being < 20 degrees at 5 min-irradiation time (hydrophilic surface). The contact angle of H_3PO_4-modified samples also decreased with the increase of irradiation time, most significantly at $\geq$ 3 min-
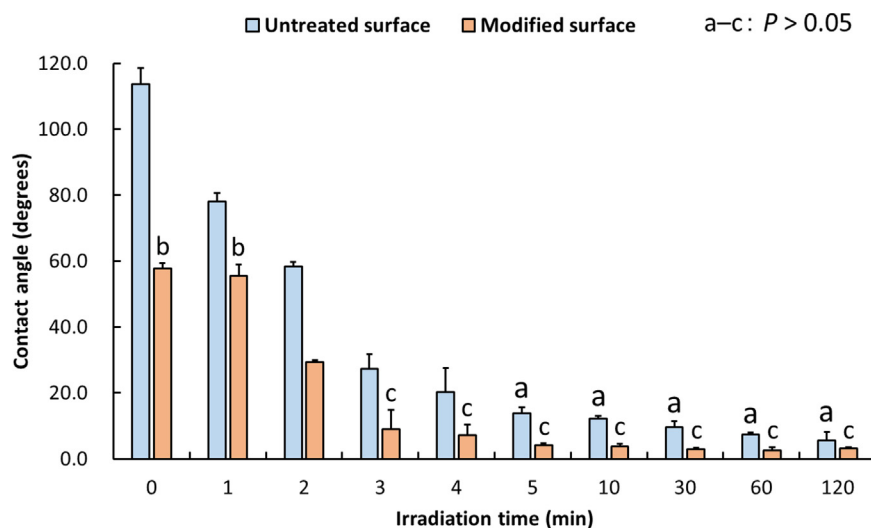
Fig. 3. Mean contact angle values in untreated and H_3PO_4-modified HA surfaces irradiated with ultraviolet/ozone (UV/O_3). The same letter (a to c) above the values indicate that there were no significant differences between the two types of samples ($P > 0.05$). The error bars indicate standard deviation.

irradiation time ($P < 0.01$). Furthermore, the contact angle of modified samples irradiated with UV/O$_3$ for 5 min after 7-day storage is shown in Fig. 4. Storage time did not affect the contact angle ($P > 0.05$).

3.2. Osteoblast-like cell response to H$_3$PO$_4$ modified HA surface decontaminated by UV/O$_3$ irradiation
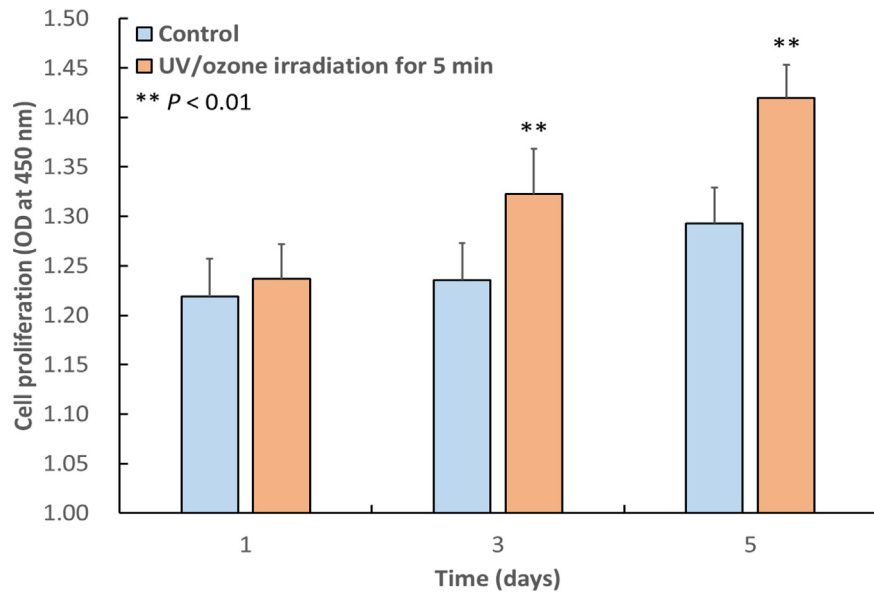
3.2.1. Cell proliferation

Proliferation of MC3T3-E1 osteoblast-like cells on control *H$_3$PO$_4$*-modified samples and on those irradiated with UV/O$_3$ for 5 min was monitored for 1, 3, or 5 days (Fig. 5). At 3 and 5 days, proliferation of cells grown on 5 min-irradiated samples were significantly higher than that of cells cultured on control samples ($P < 0.01$).

### 3.2.2. Cell differentiation and mineralisation

Relative mRNA levels of the *ALP* and *OCN* genes were determined in MC3T3-E1 osteoblast-like cells cultured for 7, 14, or 21 days after obtaining a confluent cell monolayer on control *H$_3$PO$_4$*-modified samples and on those irradiated with UV/O$_3$ (Figs. 6 and 7). At days 7 and 14, the expression levels of *ALP* and *OCN* in cultures grown on irradiated samples were numerically higher than those in cultures grown on control samples, but the effect did not reach statistical significance ($P > 0.05$). At 21 days, the expression levels of *ALP* and *OCN* in cells grown on irradiated samples were significantly higher than those in cells cultured on control samples ($P < 0.01$, $P < 0.05$, respectively).
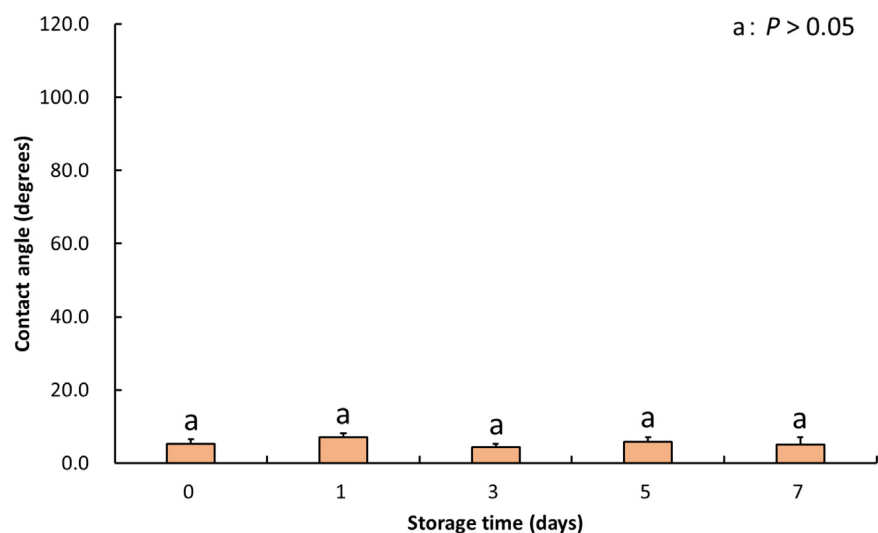

Fig. 4. Mean contact angle values in H$_3$PO$_4$-modified HA surfaces irradiated with UV/O$_3$ for 5 min throughout 7-day storage. No effect of storage duration on the contact angle was observed ($P > 0.05$). The error bars indicate standard deviation.

Fig. 5. Mean OD_{450} values proportional to the extent of proliferation of MC3T3-E1 osteoblast-like cells cultured for 1, 3, or 5 days on control H_3PO_4-modified HA surfaces and on modified HA surfaces irradiated with UV/O_3 for 5 min. At 3 and 5 days, proliferation of cells grown on irradiated surfaces was significantly higher than that of cells cultured on control surfaces ($P < 0.01$). The error bars indicate standard deviation.
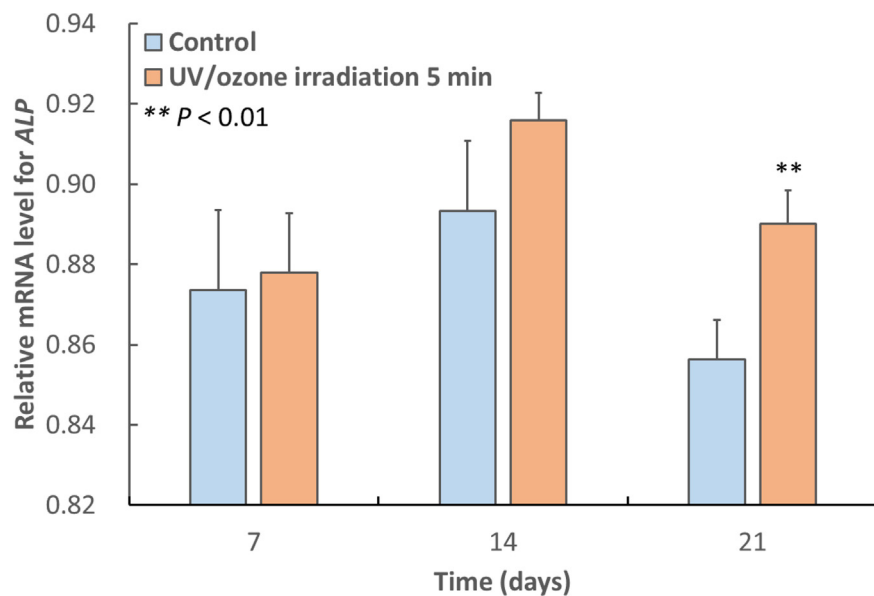
Fig. 6. Mean relative *ALP* mRNA levels in MC3T3-E1 osteoblast-like cells cultured for 7, 14, or 21 days after obtaining a confluent cell monolayer on control H_3PO_4-modified HA surfaces and on modified HA surfaces irradiated with UV/O_3 for 5 min. At 21 days, the expression on the 5 min-irradiated surface was significantly higher than that on the control ($P < 0.01$). The error bars indicate standard deviation.
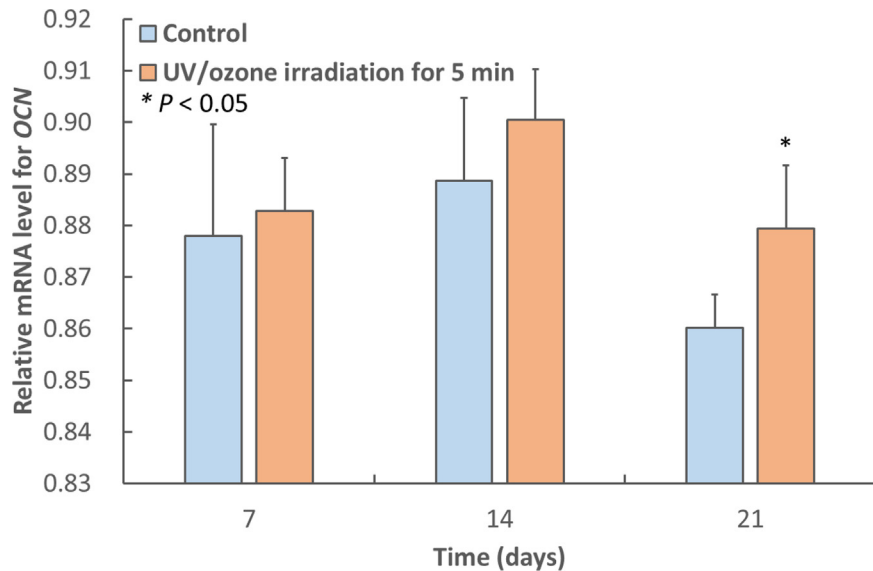
Fig. 7. Mean relative *OCN* mRNA levels in MC3T3-E1 osteoblast-like cells cultured for 7, 14, or 21 days after obtaining a confluent cell monolayer on control H_3PO_4-modified HA surfaces and on modified HA surfaces irradiated with UV/O_3 for 5 min. At 21 days, the expression on the 5 min-irradiated surface was significantly higher than that on the control ($P < 0.01$). The error bars indicate standard deviation.
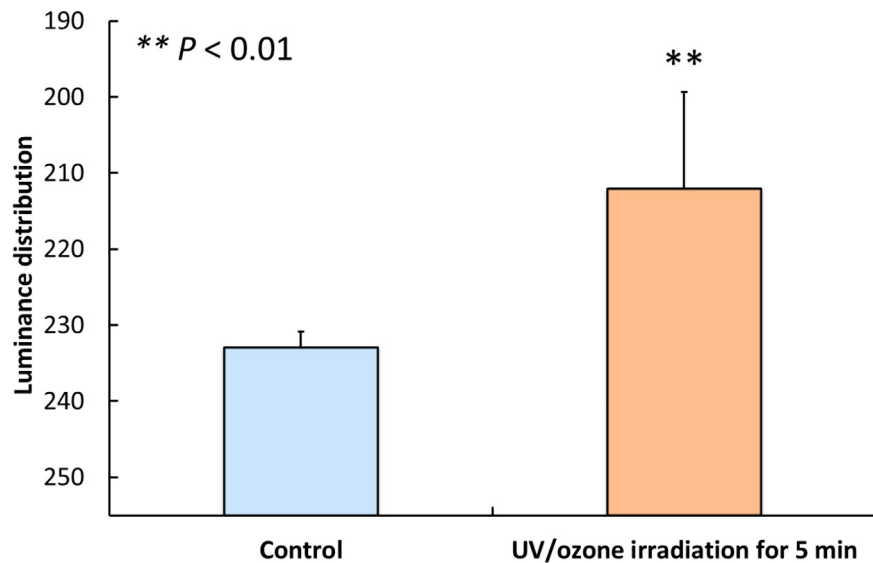
The luminance distribution of alizarin red S staining of cells grown on control and UV/O_3-irradiated H_3PO_4-modified samples at 28 days is shown in Fig. 8. The luminance distribution value in cells cultured on irradiated samples (212.1 ± 12.7) was significantly lower than that in cells grown on control samples (240.0 ± 2.1) ($P < 0.01$). Because the staining intensity is inversely proportional to the distribution value, mineralisation of cells grown on irradiated samples was significantly higher than that of cells cultured on control samples ($P < 0.01$).

4. Discussion

HA is considered as a promising material for bone tissue engineering and because its interactions with live cells are a complex, multi-step process, in this study, we examined the effects of UV/O_3-irradiation, a recognised decontamination technique, on growth parameters of osteoblast-like MC3T3-E1 cells.

4.1. Validation of the UV/O_3 irradiation method

Ra surface roughness values of untreated and H_3PO_4-modified samples were not significantly affected by UV/O_3 irradiation. Furthermore, no significant differences in Ca/P ratios were observed between irradiated and control samples ($P > 0.05$). Therefore, UV/O_3 irradiation did not alter fine morphological and chemical structure of HA surfaces.

Fig. 8. Mean values of luminance distribution of alizarin red S staining in MC3T3-E1 osteoblast-like cells cultured for 28 days on control H_3PO_4-modified HA surfaces and on modified HA surfaces irradiated with UV/O_3 for 5 min. Because the staining intensity is inversely proportional to the distribution value, mineralisation of cells grown on irradiated HA surface was significantly higher than that of cells cultured on control surface. ($P < 0.01$). The error bars indicate standard deviation.

The at. % values of C in untreated samples decreased significantly (to less than 10%) when UV/O_3 irradiation lasted 10 min or longer compared to that in non-irradiated control samples ($30.6 \pm 2.3\%$; $P < 0.05$; Fig. 2). Similarly, at. % values of C in H_3PO_4-modified samples decreased significantly upon UV/O_3 irradiation for ≥ 5 min (to $< 10\%$) compared to that in non-irradiated control samples ($24.8 \pm 1.0\%$; $P < 0.05$). Thus, UV/O_3 irradiation effectively removed the contamination from both untreated and H_3PO_4-modified HA surfaces.

As H_3PO_4-modified surface was etched and washed during sample preparation, its at. % of C value was lower than that of untreated surface. The contact angle of untreated samples decreased with the increase of irradiation time, most significantly if irradiation lasted for 5 min or longer ($P < 0.01$). Similarly, the contact angle of the H_3PO_4-modified sample decreased with the increase of irradiation time, most significantly at ≥ 3 min-irradiation time ($P < 0.01$). Notably, the contact angle of H_3PO_4-modified samples was less than 5 degrees at ≥ 5 min-irradiation time, indicating that the modified surface had become superhydrophilic. In our previous study [7], we found that the modified surface becomes hydrophilic following morphological and chemical modifications. In both the previous and current studies, we used deionized water rather than culture medium for the evaluation of wettability because the latter includes ions that may be absorbed on HA surface. Subsequent UV/O_3 irradiation likely removed these contaminants from the surfaces, most of which were hydrophobic in character, resulting in the

creation of the superhydrophilic surface. In the present study, no change in wettability of H_3PO_4-modified surface irradiated with UV/O_3 for 5 min was observed over 7-day storage ($P > 0.05$). Although Noro et al. [14] reported that surface wettability of UV-treated zirconium was maintained after immersion in an aqueous condition, here we showed that the wettability of H_3PO_4-modified surface irradiated with UV/O_3 for 5 min was maintained also after dry storage. These findings demonstrate that UV/O_3 irradiated surfaces are less likely to become re-contaminated.

4.2. Osteoblast-like cell response H_3PO_4-modified HA surface decontaminated by UV/O_3 irradiation

Proliferation of MC3T3-E1 osteoblast-like cells on H_3PO_4-modified HA surface irradiated with UV/O_3 was significantly higher than that on control surfaces at 3 and 5 days ($P < 0.01$). The expression of the *ALP* and *OCN* genes in the cells grown on irradiated HA surface was significantly higher than that in cells cultured on control HA surface at 21 days ($P < 0.01$ and $P < 0.05$, respectively). Furthermore, at 28 days, alizarin red S staining intensity of cultures grown on irradiated HA surface was significantly higher than that of the cells grown on control HA surface ($P < 0.01$). These results suggest that decontamination by UV/O_3 irradiation promoted cell proliferation and accelerated differentiation and mineralisation.

Ducheyne and Qiu [15] reported that the following events occur at the bioactive ceramics-tissue interface prior to cell attachment and proliferation: (1) dissolution from the ceramics; (2) precipitation from the solution onto ceramics surface; (3) ion exchange and structural rearrangement at the ceramics-tissue interface; (4) interdiffusion from the surface boundary layer into the ceramics; (5) solution-mediated effects on cellular activity; (6) deposition of either the mineral phase or the organic phase; and (7) chemotaxis to the ceramic surface. Furthermore, Bertazzo et al. [6] reported that the equilibrium between modified HA surface and biological fluids leads to protein adsorption and cell adhesion. We suggest that the decontamination of HA surface accelerated the emergence of the equilibrium state as a result of the direct contact at the HAP-tissue interface.

We believe that in our current experiments, dissolution of Ca^{2+} ions was activated at the interface and the increased amount of Ca^{2+} ions led to an acceleration of the differentiation and mineralisation of MC3T3-E1 cells. In particular, Wang et al. [16] demonstrated that a calcium phosphate ceramics substrate supported osteoblast growth and bone-related gene expression and that the characteristic gene expression pattern explained the basis of the biocompatibility and bioactivity observed for calcium phosphate ceramics. Additionally, Chang et al. [17]

suggested that an elevated concentration of calcium and phosphate is crucial for *in vitro* mineralisation.

Furthermore, we also suggest that the effects of the combination of acid etching and UV/O$_3$ irradiation on HA surfaces developed in the following steps: (1) surface modification of HA by an acid-etching procedure leading to high solubility because of a reduction in Ca/P ratio; (2) removal of surface contaminants by UV/O$_3$ irradiation; (3) emergence of a more direct surface contact with the culture medium; (4) activation of ion exchange at the interface; (5) earlier attainment of the equilibrium state; and (6) acceleration of the proliferation, differentiation, and mineralisation of MC3T3-E1 cells.

5. Conclusion

In this study, we established a UV/O$_3$ irradiation method for HA surfaces treated with 30% phosphoric acid solution and evaluated *in vitro* response of MC3T3-E1 cells to the modified HA surface decontaminated by UV/O$_3$ irradiation. UV/O$_3$ irradiation of the modified surface for ≥ 5 min significantly decontaminated the surface, improved its wettability, and accelerated the proliferation, differentiation, and mineralisation of MC3T3-E1 cells. Therefore, UV/O$_3$ irradiation of HA as a scaffold will likely serve as a clinically beneficial method to provide highly biocompatible HA-containing products to patients.

Declarations

Author contribution statement

Keisuke Yasuda: Performed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper

Yohei Okazaki: Performed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Yasuhiko Abe: Conceived and designed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kazuhiro Tsuga: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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