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SLA 티타늄 표면에서 시간 조건에 대한 자외선 조사의 광기능화

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I. 서론

1965년 티타늄 임플란트가 임상에서 처음으로 적용된 이후 고정체의 디자인, 표면처리에 대한 연구를 통해 현재 치과용 임플란트의 10년 생존율은 96% 이상으로, 임플란트 식립술은 임상적으로 매우 예지성 높은 술식이다. 그럼에도 불구하고 임플란트와 골계면간의 접촉률은 50-65% 수준이며, 골질이나 골양이 부족한 고령층이나 골대사와 관련된 전신질환 환자 등에서도 임상적으로 안정적인 결과를 얻기 위해서는 임플란트 골유착을 증진시키고 임플란트와 골계면과의 접촉률을 증가시킬 필요가 있다.

티타늄은 가공된 직후에 자연 산화막이 형성되는데, 이는 골유착(osseointegration)에 있어 매우 중요한 요소이다. 가공된 후 보관되는 과정에서 티타늄 표면은 공기 중의 탄소 오염원인 탄화수소의 흡착에 의해 산화막이 오염되고, 티타늄 표면에너지가 변함으로써 골형성세포의 부착능력이 저하되는 생물학적 활성의 감소, 즉 티타늄의 생물학적 노화(biological aging)가 발생한다. 티타늄의 노화는 표면의 소수성(hydrophobic)을 야기하며, 소수화된 티타늄 표면은 조직액 또는 혈액에 대한 젖음성이 매우 낮아 티타늄 표면으로 골모세포의 유입 및 단백질 부착을 방해함으로써 골유착 및 골계면간의 접촉률을 감소시킨다.

티타늄 표면의 친수성(hydrophilicity)을 높이기 위한 다양한 방법 중 자외선(ultraviolet) 조사를 통한 광기능화(photofunctionalization)는 표면의 세포부착, 분화, 골의 무기질화를 증가시키는 것으로 보고되고 있다.

II. 목적

이 연구의 목적은 SLA 처리된 티타늄 디스크에 다양한 조건으로 자외선 광조사를 시행한 후 시간에 따른 친수화 및 재소수화(rehydrophobization)에 대해 기계적, 화학적 특성을 분석하고 세포부착, 세포증식에 미치는 영향을 평가하고자 함에 있다.

III. 재료 및 방법

SLA 처리된 티타늄 디스크를 제작 후 8주간 상온의 멸균배지에 방치하였으며 대조군과 3개의 실험군으로 나누어 자외선을 조사하였다; 1) 대조군: 자외선 조사를 시행하지 않음, 2) 실험군1: 24시간 동안 자외선을 조사, 3) 실험군2: 24시간 동안 자외선 조사 후 24시간 동안 상온의 멸균배지에 방치, 4) 실험군3: 24시간 동안 자외선 조사 및 24시간 동안 상온의 멸균배지에 방치 후 24시간 동안 자외선 재조사.

티타늄 디스크 표면의 형상 변화를 전계방출주사전자현미경(FE-SEM)으로 분석하였고, X-선 광전자 분광법(XPS)을 통해 디스크 표면 원소의 화학적 조성과 결합 특성을 관찰하였다. 표면의 젖음성을 평가하기 위해 접촉각을 측정하였다. MC3T3-E1 세포를 티타늄디스크에 배양하여 24시간 이후 live/dead cell assay를 통해 세포의 증식에 미치는 영향을 비교하였으며 형광중첩사진을 통해 세포골격을 관찰하여 세포부착에 미치는 영향을 비교 평가하였다. 또한, 배양된 MC3T3-E1 세포의 alkaline phosphatase (ALP) activity를 측정하여 초기 세포 분화를 평가하였고 Alizarin-red assay를 통해 초기 세포의 광화정도를 평가하였다.

IV. 결과

FE-SEM 결과에서 자외선을 조사한 실험군1, 실험군2, 실험군3과 자외

선을 조사하지 않은 대조군 사이에 디스크 표면의 구조적 변화는 관찰되지 않았다. 대조군의 접촉각은 108.4° 로 측정된 반면, 실험군1, 실험군2, 실험군3의 접촉각은 각각 1.85° , 1.48° , 1.18° 로 감소하였다. 이를 통해 자외선을 이용한 티타늄의 광기능화는 표면의 친수성을 증가시키는 것을 확인할 수 있었다. XPS를 통해 티타늄 표면 원소를 분석한 결과, 대조군에서 29.42%의 탄소가 측정되었으나 실험군1, 실험군2, 실험군3에서는 17.2%, 17.28%, 19.5%로 관찰되어 자외선을 조사한 모든 실험군에서 유의한 탄소량의 감소를 보였다. 산소는 대조군에서 53.37%, 실험군1에서 62.36%, 실험군2에서 62.68%, 실험군3에서 60.95%로 관찰되었으며, 대조군에 비해 모든 실험군에서 유의한 산소량의 증가를 보였으나 실험군 사이의 유의한 차이는 관찰되지 않았다. 세포배양 24시간 이후 live/dead cell assay 결과 실험군 모두에서 유의미한 차이가 관찰되지 않았으며 형광중첩사진을 통하여 세포골격을 확인한 결과 실험군간의 세포골격의 차이가 관찰되지 않았다. 또한, 세포배양 7일과 14일 후에 측정한 실험군들간의 ALP의 활성도는 유의미한 차이를 보이지 않았으며 Alizarin red 염색을 이용한 티타늄 표면의 골아세포 칼슘 침착량 분석 결과 자외선 조사를 시행한 실험군 모두에서 칼슘침착 정도의 유의미한 차이는 관찰되지 않았다.

V. 결론

생물학적 노화가 진행된 티타늄 표면에 자외선 조사를 이용한 광기능화는 티타늄 표면의 물리적 특성을 변화시키지 않으면서 표면에 축적되는 탄화수소의 양을 감소시키고 표면의 젖음성을 증가시킴을 확인할 수 있었다. 또한, 친수화된 표면은 반복되는 자외선 조사나 단기간의 재소수화 과정에서도 친수성 및 향상된 세포 분화, 부착능력과 골 침착능력을 유지하고 있음을 확인할 수 있었다. 티타늄 표면의 자외선 조사를 통한 광기능화는 임플란트의 빠른 골개조와 조기 골유착을 증진시킬 수 있을 것으로 사료된다.

Key words: Biocompatibility, Biological aging, Contact angle, Dental implants, Hydrocarbons, Hydrophilicity, Morphology, Osseointegration, Physicochemical.

I. Introduction

Titanium has advantages including high biocompatibility, excellent strength, low toxicity to fibroblasts and macrophages, and low inflammatory response to peri-implant tissues.[1] Especially, titanium forms a thin oxide layer (mainly titanium dioxide (TiO_2)) on its surface due to its high affinity for oxygen, which increases corrosion resistance. Because of this property, it is widely used in orthopedic and dental fields.

Dental implants with titanium have been used for over 50 years and the implant-supported prostheses are now accepted as highly predictable treatment options for the rehabilitation of edentulous patients, with a 10-year survival rates of 96.4%.[2] However, implant failure may increase in elderly patients who have insufficient bone quality and quantity, or in patients with uncontrolled diabetes, osteoporosis, or systemic diseases associated with bone metabolism which may impair bone healing.[3]

Osseointegration has been defined at multiple levels,[1] it is histologically characterized as the direct structural connections between bone and surface of an implant without the interposition of soft tissue.[4] Successful osseointegration between a titanium implant and bone is important to ensure excellent primary stability and functional loading following implant placement. Osteoblasts migrate to a titanium surface immediately after implant placement and differentiate to form new bone, thereby allowing a stable mechanical and biological fixation of the implant.[5] The biologic fixation is tissue anchorage by distance and contact osteogenesis of early peri-implant trabecular bone formation at 10 to 14 days after implant placement which differs from mechanical primary stability during the implant placement.[1,6] As osseointegration progress which new trabecular bone remodels into the lamellar bone in direct contact,[1] the contact area between bone and titanium

surface increases. Bone-to-implant contact (BIC), which is the ratio of an implant-bone contact area at the microscopic level, is used as a measure of osseointegration.[7] A higher BIC value is essential to achieve successful early implant osseointegration.

Various surface modifications including mechanical, chemical, and physical methods have been studied to achieve the desired biological response for early osseointegration and improvement of BIC.[6,8-10] Moreover, research in recent decades has focused on the osteoinductive potential of implant surfaces rather than the implant geometry.[6] The surface modifications increase wettability, improve cell adhesion and proliferation, and these play important roles in the long-term stability of the implant by increasing early integration of titanium into the bone.[6,11] Despite various titanium surface modifications, the BIC of implants remains far below 100%. Time-related biological degradation of titanium has been recently reported as one of the factors causing low BIC.[12] Four-week-old titanium surfaces showed reduction of biological capabilities such as adhesion and proliferation ability of osteogenic cells up to 50% compared to new titanium surfaces, and their integration into the bone was measured below 50% in an animal model.[12] This time-dependent degradation of bioactivity is referred to as biological aging. Although the mechanism of these time dependent degradation of biological activities of titanium is still unclear, it is assumed to result from a decrease in hydrophilicity and reduced cell proliferation, differentiation, and attachment due to organic contaminants such as hydrocarbons that accumulate on the titanium surface in process of time.[12] Since commercially available titanium implants are packaged in the gas-permeable state, the biological aging of titanium implants is inevitable.

Photofunctionalization of a titanium surface using ultraviolet (UV) irradiation has been introduced as an efficient method to improve the biological capabilities of titanium implants.[13] Photofunctionalization removes

the hydrocarbons which accumulate on a titanium surface through photocatalytic and photolytic activities without changing the topography of the titanium surface.[13] It converts the hydrophobic surface to a superhydrophilic surface to promote cell differentiation, proliferation, and bone mineralization and improve osteoconductivity.[13] In addition, photofunctionalization converts a titanium surface from electronegative to electropositive to improve the attachment of blood proteins and extracellular matrices to the titanium surface, thereby promoting osseointegration.[14] *In vivo* study, photofunctionalized implants have shown a greater than three-fold increase in stability of implant compared to irradiated implants in the early healing phase and had near 100% BIC.[13] A recent clinical trial also reported that photofunctionalization of titanium implants reduces the healing period following implant surgery by approximately 50%.[15]

However, numerous studies related to photofunctionalization evaluated the biological activity of a titanium surface immediately after photofunctionalization and there is little study of the duration of the effect of UV irradiation.[15] Miyauciet al. reported that the effect of UV irradiation lasted just for a few minutes immediately after photofunctionalization.[16] This short-lasting effect of UV irradiation inconveniences operators as they must continuously irradiate an implant fixture until just before surgery in clinical settings. Furthermore, research on the effect of re-photofunctionalization on a titanium surface that was re-hydrophobized after photofunctionalization is rare. The objective of this study was to compare the time-related modifications associated with physicochemical and biological characteristics through short-term re-hydrophobization and UV re-irradiation of titanium surfaces.

II. Material and Methods

1. Titanium disc samples

In this study, titanium discs (10.0 mm in diameter and thickness of 1.0 mm) were used for surface characterization analysis. For *in vitro* study, another larger titanium discs were prepared (20.0 mm in diameter and thickness of 1.0 mm). All surfaces of discs were hydroxyapatite-sandblasted and acid-etched with hydrochloric/sulfuric acid (KJ Meditech Company, Gwangju, South Korea). After manufacturing titanium discs, all discs were stored in sterilized sealed containers for 8 weeks to allow enough biological aging.

2. UV light irradiation

Photofunctionalization was performed with four 15W bactericidal lamps (G15T8, Sankyo Denki, Tokyo, Japan) at an intensity of 5.0 mW/cm^2 (wavelength = 254 ± 20 nm). The duration of the UV irradiation was as follows: 1) Control group: No UV irradiation; 2) Group 1: UV irradiated for 24 hours; 3) Group 2: UV irradiated for 24 hours and then stored in an ambient sterilized medium; and 4) Group 3: UV irradiated for 24 hours followed by storing for 24 hours in an ambient sterilized medium and then UV re-irradiated for 24 hours. Four groups were compared for surface characterization analysis. An *in vitro* experiment was conducted among the experimental groups to assess the changes in the bioactivity of titanium surfaces under different UV irradiation conditions.

3. Surface analysis of the samples

Field emission scanning electron microscopy (FE-SEM, S-4800, Hitachi, Tokyo, Japan) was performed to assess the effect of photofunctionalization on titanium surface morphology.

To assess the wettability of the disc surfaces, contact angles were measured using a goniometer (GS, Surface Tech Co. Ltd., Gwangju, South Korea) with the sessile drop method.

The chemical compositions of the titanium disc surfaces were analyzed using X-ray photoelectron spectroscopy (XPS, K-Alpha, Thermo VG Scientific, East Grinstead, West Sussex, UK). We evaluated the titanium, carbon, and oxygen atomic contents using a monochromatic Al K-Alpha anode X-ray source (hv = 1486.8 eV of K-Alpha radiation was used). A fitting procedure was done to analyze the shape of core lines using Avantage software (ThermoFisher Scientific, Waltham, MA, USA).

4. Cell culture

MC3T3-E1 (CRL-2593, ATCC, Manassas, VA, USA), a clonal pre-osteoblastic cell derived from mouse calvaria, was cultured in an α-modified minimum essential medium (α-MEM) supplemented with a penicillin-streptomycin solution and 10% fetal bovine serum. Cells were incubated in a CO_2 incubator containing 5% CO_2 mixed gas at 37°C.

5. Cell viability

Titanium discs were placed in 12-well plates, and $5x10^4$ cells were seeded onto the plates. After 24 hours, phosphate buffered saline (PBS) was used to wash the plates. And then, the cells were stained using a live/dead cell kit (BioVision #501-100, BioVision Inc., Milpitas, CA, USA). 0.25 µL of Solution A and 1 µL of Solution B were mixed per 1 mL of staining buffer. The cells were incubated with the mixed solution in an incubator at 37°C for 15 minutes. The cells were examined under a fluorescence microscope at 40x and 100x magnification.

6. Cell morphology

Titanium discs were placed in 12-well plates, and $5x10^4$ cells were seeded onto the plates. After 24 hours, cell fixation was done in 4% formaldehyde for 15 minutes. The cell washing was done twice with PBS to remove residual formaldehyde and treated with 0.1% Triton X-100 for 10 minutes for permeabilization. Subsequently, the cell washing was done twice using PBS and blocked with 1% bovine serum albumin in PBS. And then, cell washing was performed twice using PBS, and 20 µL of phalloidin (VECTASHIELD®, H-1600, Vector Laboratories, Burlingame, CA, USA) and 10 µL of DAPI (VECTASHIELD®, H-1200, Vector Laboratories, Burlingame, CA, USA) were mixed per 1 mL of PBS. The cells were incubated with the mixed solution for 15 minutes. The cells were then examined under a fluorescence microscope (NI-SS, Nikon, Tokyo, Japan) at 400x magnification.

7. Evaluation of ALP activity and bone calcification

Titanium discs were placed in 12-well plates, and $1x10^5$ cells were seeded onto the plates. The cells were differentiated for 14 days by adding a differentiation medium. The culture medium was changed every two days and contained α-MEM, 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 100 uM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), and 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). An alkaline phosphatase (ALP) assay was performed after 7 and 14 days. Cell washing was performed twice using dPBS and incubated with a 0.9% NaCl solution containing 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes. Following lysis, the cells were scratched off from the plate, and their lysate was centrifuged at 2,500×g and 4℃ for 10 minutes. The lysate was then incubated with a p-nitrophenyl phosphate solution (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C for 30 minutes. After blocking the reaction by adding 600 µL of 1.2N NaOH, ALP activity was measured by the absorbance at 405 nm using an ELISA reader and quantified in terms of protein concentrations. Data were expressed in µmole p-NP/min/µg of protein.

Alizarin red S staining was performed 7 and 14 days later to evaluate the mineralization capability of cells. The differentiated cells were fixed in 4% formaldehyde for 15 minutes, cell washing was performed twice using dPBS, and stained with 1% Alizarin red S (pH 4.2) for 15 minutes. Cell washing was performed twice using dPBS. They were then eluted with 10 mM sodium phosphate and 10% cetylpyridinium chloride for 15 minutes. Absorbance was measured at 560 nm using an ELISA reader.

8. Statistical analysis

The data were expressed as mean ± standard deviation. Statistical analysis was performed by SPSS Version 20.0 (SPSS Software, Chicago, IL, USA). A Kruskal-Wallis test was used to analyze the effects of the different UV irradiation conditions, while $p<0.05$ was considered statistically significant.

Ⅲ. Results

1. Morphology of the titanium surfaces

Based on FE-ESM results, UV irradiated discs showed no noticeable differences in roughness and did not affect the surface morphology of SLA titanium disc. Macro and micro roughness were observed on the disc surfaces in all four groups as typically seen on SLA surfaces. As shown in the 5,000x magnification SEM image in Figure 1, the UV irradiated and unirradiated titanium surfaces were abundant in pores measuring 1-10 um in diameter.

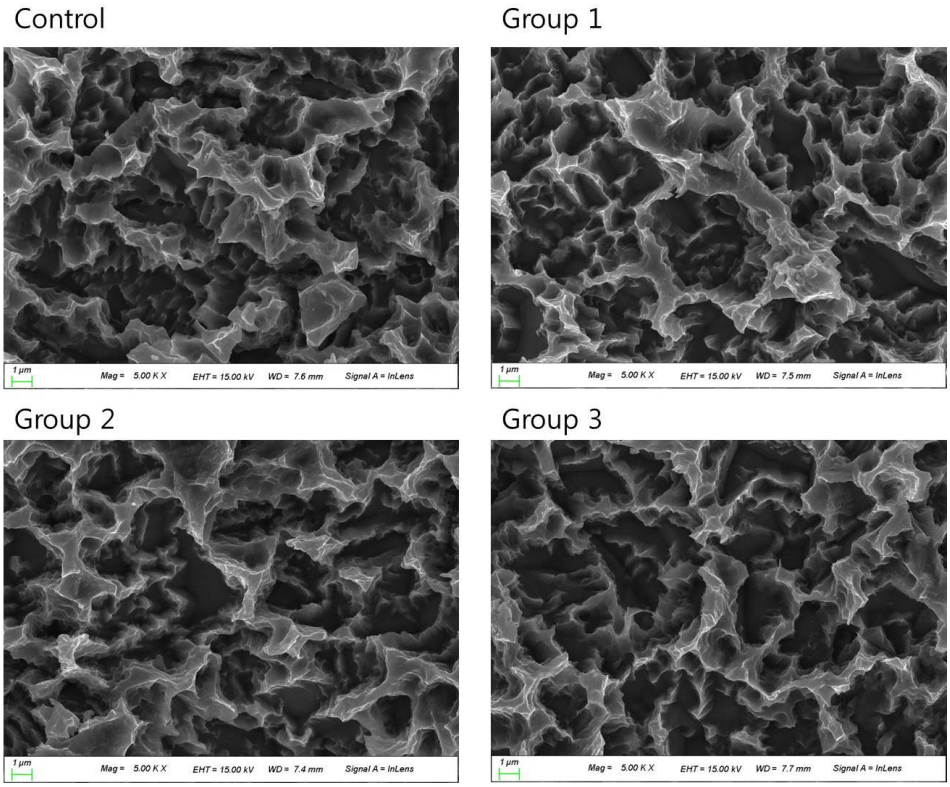

Figure 1. FE-SEM images of control and experimental groups. No difference of the surface roughness was observed between unirradiated surface and UV irradiated groups (x 5,000). Scale bar indicates 1 um. (Control group: unirradiated titanium surface, Group 1: UV irradiated titanium surface for 24 hours, Group 2: UV irradiated for 24 hours and then stored in an ambient sterilized medium, Group 3: UV irradiated for 24 hours followed by storing for 24 hours in an ambient sterilized medium and then UV re-irradiated for 24 hours)

2. Contact angle

Figure 2 shows the contact angle with images of the droplets. The contact angle on unirradiated titanium surface was 108.4°, indicating hydrophobicity. UV light irradiation significantly enhanced surface hydrophilicity. After UV irradiation, the water contact angles were decreased to 1.85°, 1.48°, 1.18° for group 1, 2, and 3, respectively. This indicated that UV irradiation changes titanium surface from hydrophobic to superhydrophilic surface. No significant differences in contact angles were found among experimental groups, indicating that the increase in hydrophilicity after UV irradiation was maintained equally for 24 hours.

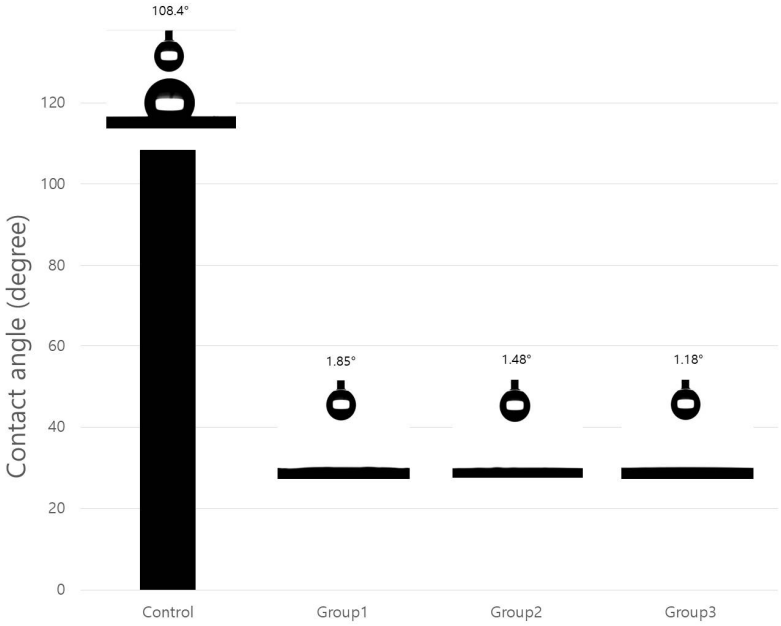

Figure 2. Contact angles of unirradiated titanium surface and UV irradiated titanium surfaces. Unirradiated titanium surface showed hydrophobic status. In contrast, UV irradiated surfaces showed superhydrophilic surface regardless of different UV irradiation conditions. (Control group: unirradiated titanium surface, Group 1: UV irradiated titanium surface for 24 hours, Group 2: UV irradiated for 24 hours and then stored in an ambient sterilized medium, Group 3: UV irradiated for 24 hours followed by storing for 24 hours in an ambient sterilized medium and then UV re-irradiated for 24 hours)

3. XPS analysis

Table 1 shows the elemental ratio of a titanium surface measured using XPS. The carbon atomic ratio significantly reduced from 29.42% on the unirradiated surfaces to 17.20%, 17.28%, and 19.50% in Group 1, 2, and 3, respectively. UV irradiation also increased the oxygen atomic ratio. No significant difference in the surface elemental ratio was found among experimental groups.

	C	O	Ti
Control	29.42%	53.37%	17.20%
Group 1	17.20%	62.36%	20.44%
Group 2	17.28%	62.68%	20.04%
Group 3	19.50%	60.95%	19.55%

Table 1. Atomic percentage of titanium surfaces

As no significant difference of the spectra of XPS and results of C1s and O1s measurements was observed between the UV irradiated groups, only the results of the control group and Group 1 are presented in Figures 3 and 4. As shown in Figure 3, the C1s peaks were reduced and the increase of O1s peaks was observed in the UV irradiated group compared to the control group. The C1s spectral profile in Figure 4a and 4b consisted of three components. Hydrocarbons (C-H) had the highest intensity of binding energy of 285 eV, and their peaks were reduced in the UV irradiated group (binding energy of 286.3 eV represents C-O-C, binding energy of 288.8 eV represents O-C=O). This demonstrated that UV irradiation can effectively remove carbon from titanium surfaces. Binding energy of 530.1 eV, represents TiO_2 peak, showed a slight increase in the UV irradiated group. The binding energy of

532.2 eV corresponded to C=O and C-C=O, which are carbon pollutants. The peaks corresponding to these pollutants decreased in the UV irradiated group (Figure 4c and 4d).

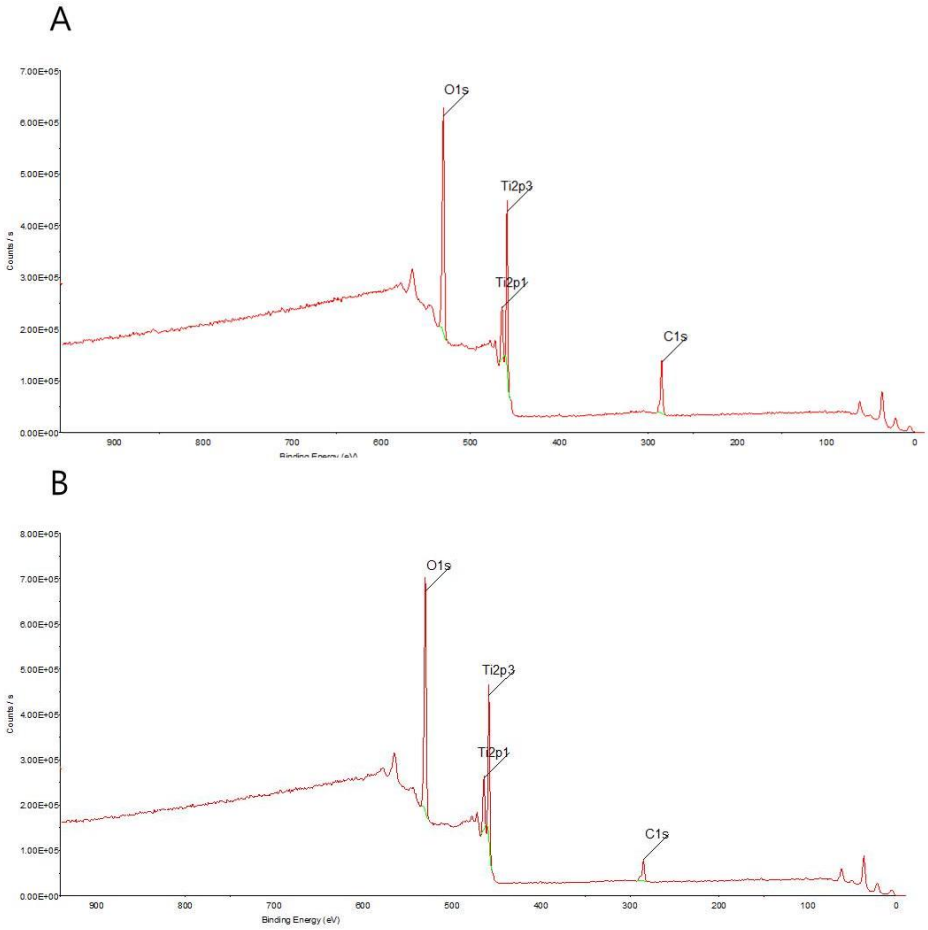

Figure 3. XPS spectra of (A) unirradiated titanium surface and (B) experimental group 1. Ti, O, C atoms were observed. Decrease of carbon peak was observed on the UV irradiated titanium surface with slight increase of oxygen peak. (Control group: unirradiated titanium surface, Group 1: UV irradiated titanium surface for 24 hours)

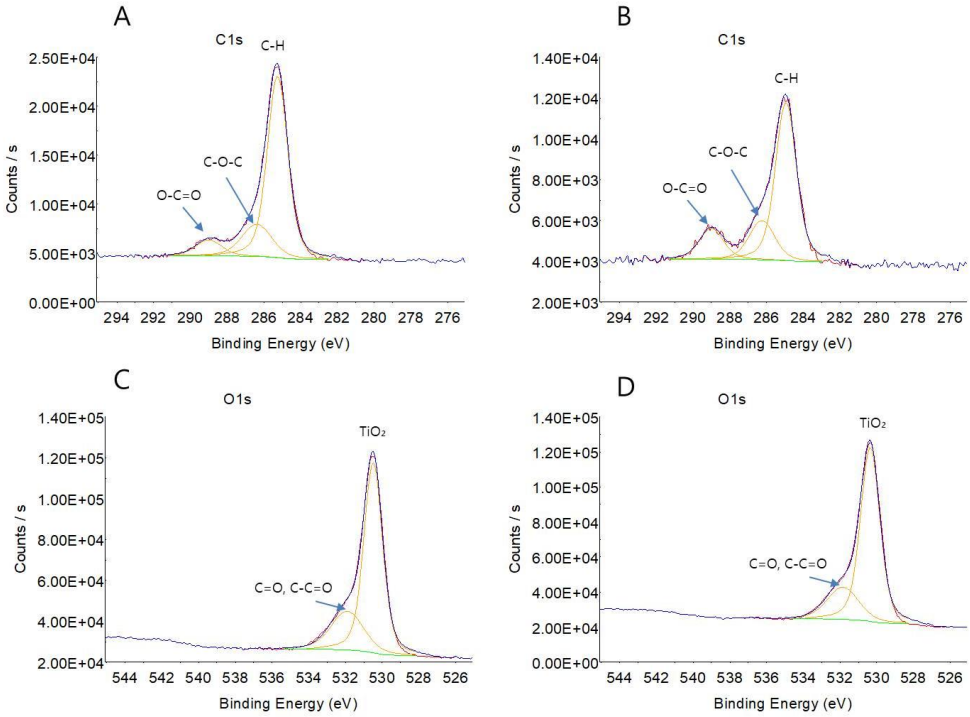

Figure 4. XPS spectra for C1s and O1s on the unirradiated Ti surface (A and C) and experimental group 1 (B and D). The peak of hydrocarbon corresponding to 285 eV decreased through UV irradiation (A and B). In O1s spectra, slight increase of oxygen peak was observed with decrease of carbon pollutants such as C=O, C-C=O (C and D). (Control group: unirradiated titanium surface, Group 1: UV irradiated titanium surface for 24 hours)

4. Cell viability and morphology

Cell viability on the titanium surfaces was estimated using live/dead double staining. No significant differences in the number of live cells (stained as green) and dead cells (stained as red) among experimental groups (Figure 5).

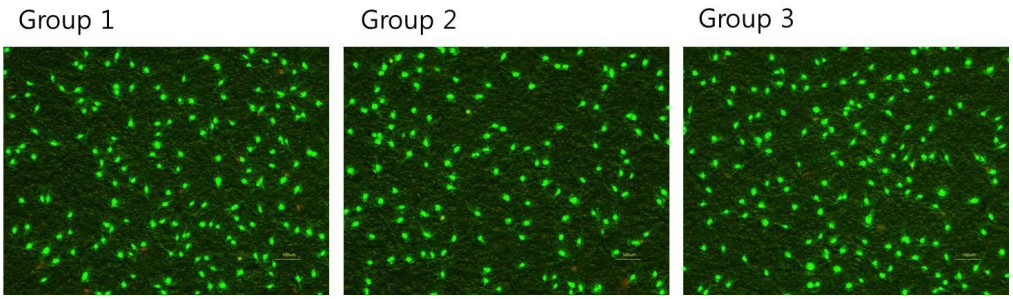

Figure 5. Live/dead cell staining images of MC3T3-E1 cells cultured on the titanium surfaces. Green spots indicate for live cells and red spots indicate for dead cells. Scale bar indicates 100 um. (Group 1: UV irradiated titanium surface for 24 hours, Group 2: UV irradiated for 24 hours and then stored in an ambient sterilized medium, Group 3: UV irradiated for 24 hours followed by storing for 24 hours in an ambient sterilized medium and then UV re-irradiated for 24 hours)

Phalloidin and 4',6-diamidino-2-phenylindole (DAPI) fluorescence staining was done to examine the morphology of the cytoskeletons and nuclei of osteoblastic cells cultured on titanium surfaces. No significant differences in the number of nuclei stained with DAPI (blue color) were found between the different UV irradiation conditions. Actin filaments stained with phalloidin (red color) extended with a similar elongated projection in multiple directions. The dynamic actin structures such as filopodia and lamellipodia indicating cell attachment ability showed no differences among experimental groups (Figure 6).

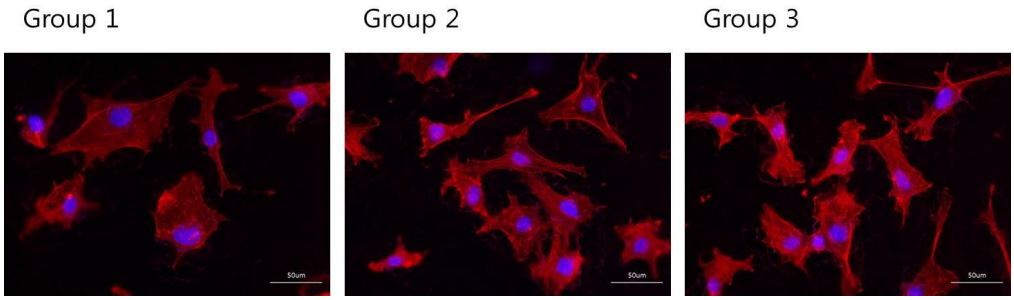

Figure 6. Fluorescent cell images on the titanium surfaces after 24 hours of cell seeding. Confocal fluorescence microscopy images with dual staining for nuclei (DAPI-blue) and actin filaments (phalloidin-red). Scale bar indicates 50 um. (Group 1: UV irradiated titanium surface for 24 hours, Group 2: UV irradiated for 24 hours and then stored in an ambient sterilized medium, Group 3: UV irradiated for 24 hours followed by storing for 24 hours in an ambient sterilized medium and then UV re-irradiated for 24 hours)

5. ALP activity

To compare the differentiation of osteogenic cell on a titanium surface, ALP activity was measured. As shown in Figure 7, no significant difference in ALP activity was found among experimental groups at 7 days after cell seeding ($p>0.05$). ALP upregulated in all experimental groups without any significant difference after 14 days in culture ($p>0.05$).

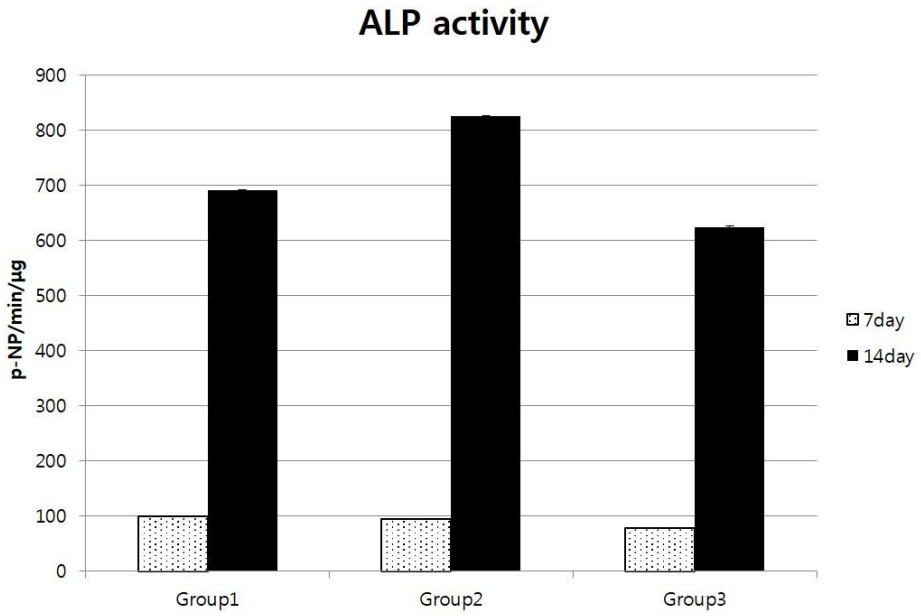

Figure 7. ALP activity of MC3T3-E1 cells at day 7 and 14 on UV irradiated titanium surfaces. (Group 1: UV irradiated titanium surface for 24 hours, Group 2: UV irradiated for 24 hours and then stored in an ambient sterilized medium, Group 3: UV irradiated for 24 hours followed by storing for 24 hours in an ambient sterilized medium and then UV re-irradiated for 24 hours)

6. Evaluation of bone calcification

At day 7 and 14 of the culture, the area of the calcium nodule was detected by Alizarin red staining to examine bone mineralization. Increased calcium deposition was observed after 14 days in culture compared to 7 days in all experimental groups. As shown in Figure 8, Group 3 had the highest total amount of calcium deposition after 7 and 14 days in culture. However, the differences in the amount of calcium deposition among experimental groups were not significant ($p>0.05$).

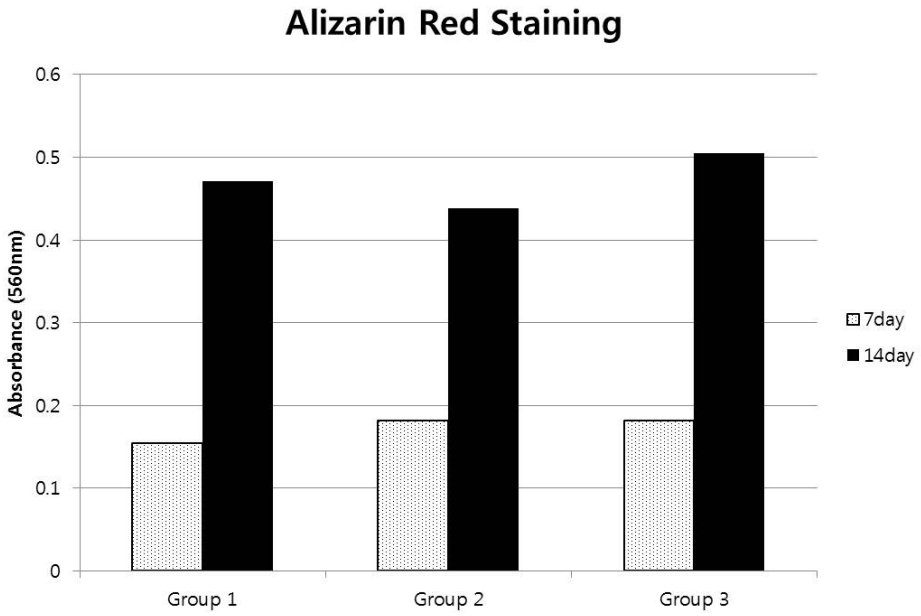

Figure 8. Quantitative calcium deposition data from Alizarin red staining experiments on MC3T3-E1 cells cultured for 7 and 14 days on the titanium surfaces. (Group 1: UV irradiated titanium surface for 24 hours, Group 2: UV irradiated for 24 hours and then stored in an ambient sterilized medium, Group 3: UV irradiated for 24 hours followed by storing for 24 hours in an ambient sterilized medium and then UV re-irradiated for 24 hours)

Ⅳ. Discussion

Biological aging of titanium means the degradation in the osteoconductivity and biological capabilities of titanium due to contamination by organic materials that accumulate on the titanium surface immediately after manufacturing.[12] Commercially available titanium implants have hydrophobic surfaces with reported ratios of hydrocarbons and other organic contaminants of 55-76%.[17,18] Over a 50% reduction in the ability of proliferation of the osteoblasts and in ALP activity which expressed differentiation ability of osteogenic cells after four weeks of manufacturing has also been reported.[12,19] In an *in vivo* experiment on a rat model, the push-in value of bone and four-week-old titanium implants was less than 50% compared to immediately installed implant after manufacturing, indicating an evident decrease in mechanical retention due to biological aging.[12] These biomechanical results showed that biological aging of titanium delays osseointegration by preventing the direct binding between bone and the titanium surface.

Many methods have been studied to overcome the biological aging of titanium.[10] Major methods include the saline storage method, atmospheric pressure plasma irradiation, polyelectrolyte coating method, and photofunctionalization by UV irradiation.[10] The saline storage method is submerging an implant in an isotonic NaCl solution immediately after acid etching. By submerging in isotonic solution, the pure surface of titanium implant can be protected from organic pollutants.[20] Saline-stored SLA implants have shown increased levels of osteoblast differentiation and BIC was approximately 20% higher than SLA implants.[20,21] However, Na and Cl in the saline solution can strongly and stably bind to titanium surfaces and negatively affect biological behaviors such as cell attachment.[22] Although

atmospheric pressure plasma irradiation forms a superhydrophilic surface and effectively improves cell attachment by removing organic contaminants from titanium surfaces and improving surface energy, plasma irradiated titanium surfaces are re-hydrophobized within a relatively short time compared to other methods.[23,24] A polyelectrolyte coating method has been recently introduced as a way to increase the hydrophilicity of titanium surfaces, however it lacks the ability to increase wettability compared to existing methods.[25]

Photofunctionalization of titanium by UV irradiation is currently one of the most representative methods to overcome the biological aging of titanium. High energy photons of UVC can break the bonds between the carboxyl group accumulated on titanium surface and titanium di-oxide surface.[26] Although the mechanism by which photofunctionalization improves the biological capabilities of titanium is unclear, it is believed that the electrostatic status of UV irradiated surfaces plays an important role.[27] Titanium surface is in an electropositive state immediately after manufacturing. However, the surface turns electronegative status as hydrocarbons accumulate. The surface returns to the electropositive state following hydrocarbon removal by photofunctionalization.[12,27,28] The bioactivity of UV irradiated titanium was found to significantly decrease after an anion masking, possibly because the positive charge on the surface following photofunctionalization was neutralized by the anion masking.[29] Since the cell membranes are negatively charged, a positively charged titanium surface is advantageous for cell attachment. Positively charged surface also facilitates the binding of PO_4^{3-} with titanium surfaces and can thus increase calcium deposition on titanium surface as well as the bonding strength with calcium[27]; this in turn, may promote apatite deposition and mineralization.[27] In addition, UV irradiation improved cell attachment on titanium surfaces by removing hydrocarbons that interfere direct bonding with proteins, thereby allowing proteins, as well as O, N, and S atoms in the cell membrane to bind easily

with the titanium surfaces.[26] Both UVA light and UVC light are useful for photofunctionalization, and UVC light was used in this study. Gao et al.[30] reported a greater improvement in the hydrophilicity and bioactivity of titanium surfaces after photofunctionalization with UVC light, likely because UVC light has a higher intensity than UVA light.

Microroughness is also an important factor for successful osseointegration. A moderately roughened surface with a roughness values (Ra) of 1-2 um made by grit-blasting and the acid-etching process seems to be optimal for the proliferation of osteoblasts and increases the mechanical interlocking between bone and titanium surfaces.[31] Through FE-SEM observation, we found that UV irradiation does not affect the microroughness of SLA surfaces, indicating that an increase in wettability and biocompatibility results from changes in the surface chemistry rather than the surface structure. The carbon ratio of the titanium surfaces of commercially available titanium implants has been measured around 50-70%.[17,18,26,32] In this study, UV irradiation was found to reduce the carbon ratio on titanium surfaces by approximately 17% in the analysis of a surface elemental composition using XPS, demonstrating that UV irradiation can reduce surface carbon ratios by up to 3-4-fold. Photofunctionalization can also reduce surface contamination by removing CF_2 components that accumulate on the titanium surface during the acid-etching procedure. The increase in the peaks on the O1s spectra indicated that photofunctionalization can improve ionization of O and Ti atoms.[26] UVC photon energy can produce hydrogen ion and reactive oxygen species such as hydroxyl radical.[33] It appears that hydroxide ions produced by UV irradiation on a titanium surface turn a hydrophobic surface into a superhydrophilic surface.[26] In this study, the wettability of a titanium surface was assessed by measuring sessile drop contact angle. The contact angle of the irradiated titanium surfaces was approximately 100 degrees, indicating hydrophobicity, whereas the contact angle for UV irradiated titanium surfaces

was measured close to 0 degrees. This dramatic decrease in the contact angle indicates that photofunctionalization can cause titanium surfaces to turn superhydrophilic. As shown by numerous studies, wettability is an important factor in cell attachment and critical for successful osseointegration,[34,35] because blood is the first biological component in contact with the titanium surface in a cascade of biological events at bone-implant interface.[1] Areid et al.[36] reported that titanium surfaces that turned hydrophilic after UV irradiation can increase the blood coagulation rate and improve wound healing and tissue integration. Increasing the contact area between the titanium surface and blood and other biological fluids by improving wettability improves cell and protein attachment.[37] In contrast, hydrophobic surfaces disturb the contact of blood with titanium surfaces leading to a decrease in cell attachment.

From cell morphological observations, actin filaments and pseudopodia consisting of microtubules and intermediate filaments stretched out further on UV irradiated titanium surfaces than on the unirradiated surfaces,[38] indicating an improvement of early attachment of osteoblast in peri-implant osteogenesis. In a study that assessed cell attachment and proliferation on titanium surfaces using the Alamar Blue assay, a higher number and density of cells were observed on UV irradiated titanium surfaces than on newly-produced titanium surfaces.[38] Photofunctionalization also accelerated the mineralization and nucleation of apatite. The amount of apatite that accumulated on UV irradiated titanium surfaces after one day of stimulated body fluid immersion was the equivalent to the one after five days of immersion on non-photofuncionalized titanium surfaces. After five days in culture, UV irradiated titanium surfaces showed significantly higher amount of calcium deposition.[27] Berglundh et al.[39] measured the push-in values of UV irradiated and unirradiated implants and observed that the push-in values of UV irradiated implants at two weeks after implant placement were the same as the push-in values of unirradiated implants at eight weeks after implant

placement. In other words, UV irradiated implants achieved bone-titanium integration four times faster than unirradiated implants. In an experiment using a rat model of type II diabetes, the push-in value of UV irradiated implants was increased by over 80% compared to that of unirradiated implants at four weeks after implant placement.[40]

Although several studies have reported that photofunctionalization improves the wettability and bioactivity of titanium surfaces, they have mostly examined the surface characteristics of titanium immediately after UV irradiation and rarely examined re-hydrophobization and re-photofunctionalization after UV irradiation. Although we observed that the improvement in bioactivity achieved by UV irradiation is maintained for 24 hours through this study, further examination with longer re-hydrophobization time is needed. Additionally, since this study assessed short-term physicochemical and biological characteristics on titanium surfaces, further *in vivo* studies of re-hydrophobization after UV irradiation in animal models are needed.

V. Conclusion

In this study, we studied the effect of time-related modifications of UV irradiation through re-hydrophobization and UV re-irradiation on aged titanium surfaces. The major findings of this study are as follows;

1. Photofunctionalization by UV irradiation did not alter the morphology of SLA titanium surfaces.

2. Photofunctionalization by UV irradiation effectively removed the hydrocarbons that had accumulated on the aged titanium surfaces and improved surface hydrophilicity.

3. The improved bioactivity after UV irradiation was maintained during short-term re-hydrophobization and repeated re-irradiation.

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