Title: **Short-range ultraviolet irradiation with LED device effectively increases serum levels of 25(OH)D.**

Authors: Daigo Morita [1], M.D.; Yoshihiro Nishida [1], M.D., Ph.D.; Yoshitoshi Higuchi [1], M.D.; Taisuke Seki [1], M.D.; Kunihiro Ikuta [1], M.D.; Hideki Asano [2]; Naoki Ishiguro [1], M.D., Ph.D.

1) Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine
Address: 65 Tsurumai-cho, Showa-ku, Nagoya City 466-8550, Japan.
Tel.: +81-52-741-2111, FAX: +81-52-744-2260

2) Development Department, NIKKISO GIKEN Co., Ltd.
Address: 4-20-3, Ebisu, Shibuya-ku, Tokyo 150-6022, Japan.
Tel.: +81-3-3443-3732, FAX: +81-3-3473-5473

Correspondence should be sent to Dr. Y. Nishida.
Department of Orthopedic Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya City 466-8550, Japan.
Tel.: +81-52-741-2111, FAX: +81-52-744-2260, E-mail: ynishida@med.nagoya-u.ac.jp

ABSTRACT

Impairment of the activities of daily living (ADL) by osteoporosis is an important concern in developed countries with a super-aging population. Vitamin D, which is a crucial molecule in bone metabolism and mainly produced endogenously with ultraviolet (UV) light exposure, is known to be insufficient in the elderly population. We used an UV Light-Emitting Diode (UV-LED) instrument generating a narrow-range wavelength to analyze the efficacy of endogenous vitamin D production. The primary purpose of this study was to examine the effects of UV irradiation at various narrow-range wavelengths using UV-LED on vitamin D supplementation. The second one was to clarify the short-term effects of UV irradiation on bone morphology in mice. Vitamin D-starved C57BL/6 female mice (n = 7 per group) were UV-irradiated (268 nm, 282 nm, 290 nm, 305 nm, and 316 nm) with 1 kJ/m^2 twice a week for 4 weeks. UV irradiation using UV-LED had significant effects on increasing serum 25(OH)D levels in all wavelength groups ($P < 0.001$, all groups) as compared to a control group. Among irradiated groups, wavelength of 316 nm had a less marked effect on 25(OH)D production compared with other wavelengths at 1 week of UV irradiation ($P < 0.05$). Levels of $1,25(OH)_2D$ were significantly increased after 4 weeks irradiation with UV-B or UV-C irradiation ($P < 0.05$). mRNA levels of vitamin D 25-hydroxylase were increased with UV-B or UV-C irradiation (268nm-305nm), significantly. Micro-CT examination revealed that short-term (4 weeks) UV-irradiation did not induce morphological change of mice in any group. This study provides essential information that narrow-range UV irradiation with LED can increase the endogenous production of vitamin D, and mRNA levels of the responsible enzyme. Although bone morphology was not altered by short-term UV irradiation in this study, an increase of serum vitamin D might improve bone morphology with long-term irradiation.

Keywords

Ultraviolet, Vitamin D, Light Emitting Diode, Osteoporosis, Bone morphology

1. Introduction

Osteoporosis is characterized by low bone mass, progressive bone loss, and microarchitectual deterioration of bone tissue, leading to enhanced bone fragility, and a consequent increase in the risk of bone fractures [1]. It is a major risk factor affecting an individual's mobility and activity. It is estimated that osteoporosis occurs in more than 35% of women after 65 years of age [2]. Severe osteoporosis can lead to fractures even with low external force, and/or repeated microfractures of vertebral bodies leaving the individual in a bedridden state, further compromising mobility function. Preventing osteoporosis is therefore an important issue in modern society, in which the population is progressively aging. A low-cost, conservative or minimally-invasive, and generally serviceable therapy for patients with osteoporosis could therefore contribute to reducing medical costs.

Vitamin D is one of the crucial molecules involved in bone metabolism. The complicated effects of vitamin D on bone and mineral metabolic activities have been reported in previous *in vitro* and *in vivo* studies. It has been demonstrated that $1,25(OH)_2D$ has roles as a potent stimulator of bone resorption in rat bone explant culture [3]. Another study revealed the dual (positive and negative) functions of vitamin D for osteoblast activities, which might be dependent on the differentiated state of the osteoblast [4]. In contrast, many studies have documented the positive roles of vitamin D in bone formation. $1,25(OH)_2D$ stimulated osteocalcin and osteopontin production [5, 6] and decreased the pool of osteoclast precursors in bone marrow [7]. Furthermore, it has been reported that treating mature osteoblasts with $1,25(OH)_2D$ led to an upregulation of osteoblast-associated genes and subsequent osteoblast differentiation [8].

Clinically, double-blinded and randomized studies of elderly subjects have demonstrated that administration of vitamin D and calcium improved bone density and reduced the subject's

bone fracture risk significantly [9-11]. In support of these previous studies, administration of vitamin D has been established to be one of the most effective treatments for osteoporosis or as an adjunct to other, currently approved treatments such as bisphosphonate or parathyroid hormone. Moreover, administration of vitamin D might prove an effective treatment for other diseases clinically and using animal models, such as decreases in muscle function and volume (sarcopenia) [12-15], cardiovascular disease [16-18], diabetes mellitus [19], asthma [20], dementia [21], and various autoimmune and hematologic diseases [22].

Vitamin D is mainly supplied endogenously, as it is reported that more than 90% of the vitamin D required for human subjects is derived from sun exposure [23, 24]. Previtamin D_3 is the first step in the metabolic pathway and is synthesized from dermal 7-dehydrocholesterol (7-DHC) after skin exposure to ultraviolet (UV) B radiation in sunlight. Previtamin D_3 then undergoes a thermochemical reaction leading to the formation of vitamin D_3 (cholecalciferol). Vitamin D_3 is transported to the liver and converted into its stored form, 25-hydroxyvitamin [25(OH)D]. After this, it is either converted into the active form 1,25-dihydroxyvitamin D, [1,25$(OH)_2$D] or the inactive form 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] in the kidneys [25, 26]. We hypothesized that in patients with osteoporosis, who have normal hepatic and renal function, efficient vitamin D supply might depend on the previtamin D_3 production at skin by exposure to UV-B. However, many persons in our super-aging society have difficulties in obtaining adequate exposure to sunlight.

Researchers (Drs. Amano and Akasaki; Nobel laureates) in our institution developed blue light-emitting diodes (LED), and the research was conducted into deep-ultra violet (UV) LED with wavelengths (250-350 nm) aimed at future medical use. A completed “NEW” UV-LED device can generate narrow-range wavelengths of UV light compared with an incandescent or a fluorescent UV light. If the effective, narrow range of the wavelength for vitamin D supply

could be identified, patients could receive only the effective wavelength of UV, and thereby eliminate unnecessary and harmful wavelengths of UV irradiation on the vitamin D supply. Thus, the purpose of this study was to examine the effects of various narrow range wavelengths with a completed UV-LED device on vitamin D supplementation in an *in vivo* animal model. In addition to the direct effects on vitamin D supply by UV irradiation, we also analyzed the effect of UV irradiation on bone morphology.

2. Material and methods

The experimental protocol was approved by the institutional review board of Nagoya University. The scheme of the experimental protocol is demonstrated in Figure 1.

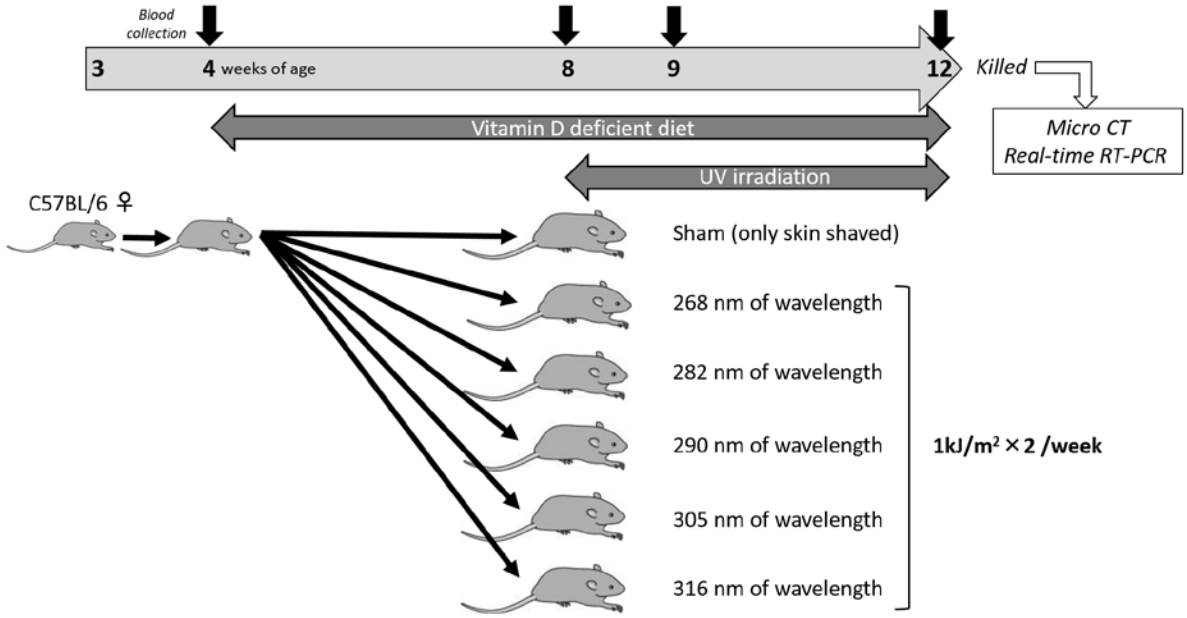


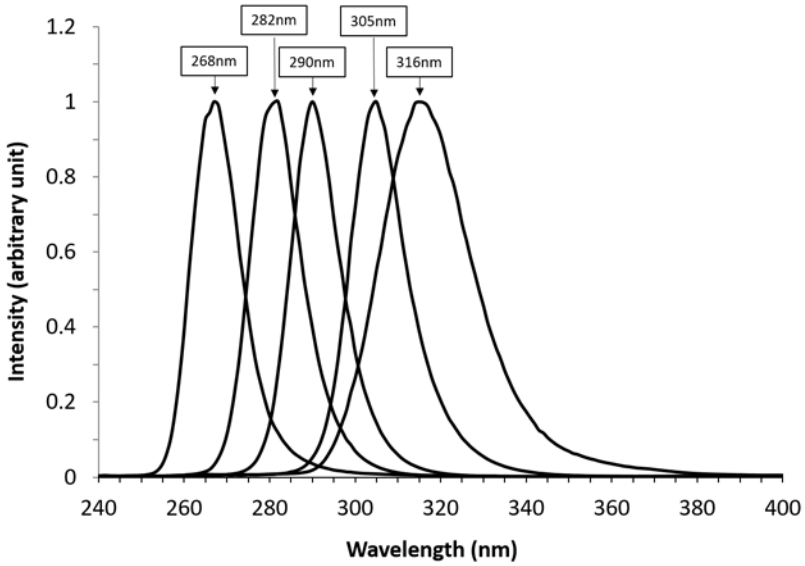
Figure 1 The experimental protocol. Inbred C57BL/6 female mice at 4 weeks of age were fed a vitamin D deficient-diet until the termination of this study (12 weeks of age). At 8 weeks of age, the mice were divided into a control group (skin was shaved, without irradiation) and five UV irradiation groups of each wavelength (268nm, 282nm, 290nm, 305nm, and 316nm). The mice were irradiated with UV irradiation regimens (1kJ/m^2 twice a week [biweekly]) from 8 weeks of age until 12 weeks of age. At 12 weeks of age, the mice were sacrificed, and specimens were obtained for experiments of micro-CT measurement or real-time RT-PCR. There were a total of six groups, with 7 mice per group.

2.1. Mice and Diet

Inbred C57BL/6 female mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). We used only female mice because female mice were more sensitive than male mice for vitamin D supplementation by UV irradiation [27]. They were kept at 25°C with a 12-h light–dark cycle and shielded from UVB. To establish a 25-hydroxyvitamin D [25(OH)D]-starved mouse colony, mice were weaned from their mothers and fed with the standard wheat-based mouse diet until 4 weeks of age. Then they were fed a vitamin D-deficient diet (AIN93GA-2, Oriental Yeast Co ltd., Tokyo, Japan) until the study termination, at 12 weeks of age [28]. AIN93GA-2 contains no vitamin D, 0.50% of calcium, and 7.00% of total fat.

2.2. UV irradiation

Surface-mounted device-packaged UV lamps of the LED system developed by Nikkiso Co ltd (Tokyo, Japan) in collaboration with Dr. Amano in our institution were used as the UV source. In order to estimate UVB effects in detail, we decided four wavelengths used on this study; the shortest and longest wavelength of UVB (280 and 315nm), and the two wavelengths to divide UVB equally among three (292 and 304nm). Furthermore, we added UVC wavelength which was apart from the shortest wavelength of UVB (265nm). Finally, after manufacturing by Nikkiso Co ltd, we applied various types of LED modules for the experiments emitting UVB (282 nm, 290 nm, 305 nm of wavelength), UVC (268 nm of wavelength), and the short wavelength end of UVA (316 nm of wavelength). The wave spectrums of each LED module were measured using a UV radiometer USR-45 DA-10 (Ushio Inc., Tokyo, Japan), and those spectrums were proved to be within a very narrow range in all modules (Fig.2).



144

145 **Figure 2 Relative spectral irradiance of prepared LED modules.** The wave spectrums and
146 intensity of each LED module (268, 282, 290, 305, and 316nm) were measured using a UV
147 radiometer under the same conditions.

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149

150 As previously described [29], a 2 × 4 cm dorsal patch of skin was clean-shaven as an area of
151 irradiation. Mice were irradiated in 4 × 6 cm compartments of a clear acrylic box. The lamps
152 were positioned 10 cm above the dorsal patch of the mice (Fig.3).

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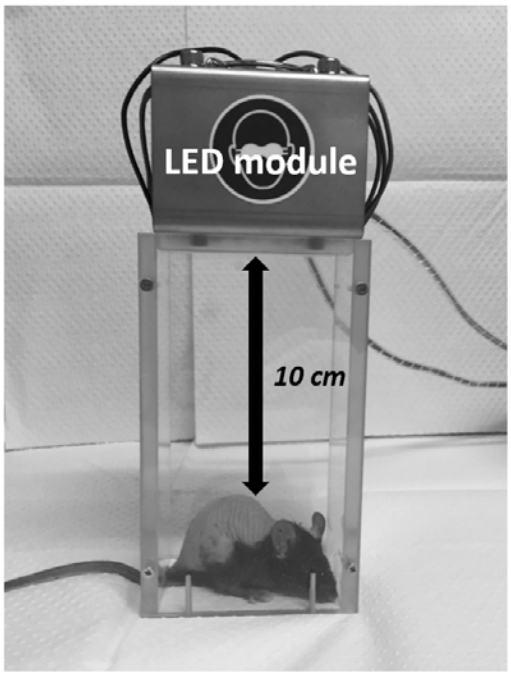


Figure 3 Scene of mouse irradiation with LED module. The LED lamps were positioned on an acrylic box 10cm above the dorsal patch where the skin was shaved for irradiation.

The radiation irradiance on the area of the dorsal patch in the box for each LED module was measured using a UV radiometer USR-45 DA-10 (Ushio Inc., Tokyo, Japan). The reflection coefficient of the box was 1.77. As for the UV irradiation dose, we decided 1 kJ/m^2 twice a week which was considered as suberythemal dose and confirmed to increase serum 25(OH)D levels [30]. We set the radiation irradiance at an identical 0.54 mW/cm^2 between groups on the basis of 268 nm wavelength with a minimum irradiance at 350 mA of the electric currents.

At 8 weeks of age, the mice were divided into a control group and five UV irradiation groups (268 nm-316 nm, 1 kJ/m^2 twice a week) [27, 30, 31]. There were a total of 6 groups, with 7 mice per group. Irradiation of 185 seconds was required for radiation irradiance of 0.54

mW/cm^2, which was equated with a dose of 1 kJ/m^2 at each wavelength. As a control group, mice were illuminated with an incandescent light for the same duration (185s) as those of the UV-irradiated mice. They were fed vitamin D deficient diets, and irradiated with UV or incandescent light from 8 to 12 weeks of age. At the age of 12 weeks, the mice were sacrificed, and samples (right femur, right kidney, and liver) were obtained. During the irradiation period, no apparent complications were observed including skin erythema.

2.3. Plasma and Serum Metabolites

At 4 weeks of age (pre-diet), 8 weeks of age (pre-UV irradiation), and 9 and 12 weeks of age (1 and 4 weeks' UV irradiation), blood samples were obtained from the plexus of the orbital vein, and stored at −20°C until quantification). Serum 25(OH)D levels and 1,25$(OH)_2$D levels were measured using RIA kits (SRL, Tokyo, Japan) following the manufacturer's protocol. The lower limit of quantification for serum 25(OH)D was 12.5 nmol/L. The vitamin D level was classified as follows: deficiency, a serum 25(OH)D concentration < 25 nmol/L; normal, 25 nmol/L ≤ 25(OH)D ≤ 90 nmol/L; sufficiency, 25(OH)D > 90 nmol/L, as described previously [31].

2.4. Real-time RT-PCR analysis

To assess the effects of UV irradiation on the regulation of the metabolism of vitamin D (25(OH)D and 1,25$(OH)_2$D), mRNA expression of enzymes, which mediates the metabolic pathway of vitamin D, was determined. Liver samples were obtained and subjected to real time RT-PCR to determine mRNA levels of vitamin D 25-hydroxylase (Cyp27a1). The kidney samples were obtained to analyze mRNA levels of 25 hydroxyvitamin D-1-alpha hydroxylase (Cyp27b1) and 1,25-dihydroxyvitamin D 24-hydroxylase (Cyp24a1).

193 Total RNA was isolated from kidney and liver of each mouse using the RNeasy Mini Kit
194 (Qiagen, Hilden, Germany) according to the protocol of the supplier. Reverse transcribed
195 cDNA was subjected to real-time RT-PCR using a LightCycler 480 (Roche Diagnostics,
196 Mannheim, Germany), with 480 SYBR Green I Master (Roche Diagnostics, Mannheim,
197 Germany), using 0.5 μM of the sense and antisense specific primers. The conventional
198 amplification program was applied; preincubation step for denaturation of the template cDNA
199 (10 min, 95°C), followed by 45 cycles of a denaturation step (10 s, 95°C), an annealing step
200 (10 s, 60°C), and an extension step (10 s, 72°C). Every run included a negative control
201 without cDNA template. To confirm the amplification specificity, the PCR products were
202 subjected to a melting curve analysis on the LightCycler 480 and also a 2% agarose/TAE gel
203 electrophoresis, to measure Tm and amplicon size, respectively. To allow relative
204 quantification after PCR, real-time efficiencies were calculated from the given slopes in
205 LightCycler 480 software (Roche Diagnostics, Mannheim, Germany) using serial dilutions.
206 The relative levels of mRNAs in a sample were expressed after normalization with those of
207 glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The primer pairs of Gapdh, Cyp27a1,
208 Cyp27b1, and Cyp24a1 primer pairs were referenced to the report by Satué et al [32].
209
210 ***2.5. Analyses with Micro-Computed Tomography (CT)***
211 The influence of altered 25(OH)D and 1,25$(OH)_2$D with UV irradiation on trabecular and
212 cortical microarchitectures of treated mice was assessed using the distal femur metaphysis at
213 12 weeks of age. Right femurs fixed in 70% ethanol were subjected to scanning with a
214 high-resolution micro-CT scanner using specific software (SkyScan 1176, SkyScan, Kontich,
215 Belgium). Briefly, each scan was performed with a source voltage of 50 kV, current of 500
216 μA, rotation step of 0.5°, and full rotation of over 180°, with a 0.5 mm aluminum filter for

beam-hardening reduction. The pixel size was 9 μm, and the exposure time was 0.89 seconds. Scans also included phantom bones for analysis of bone mineral density (250 mg/cc and 750 mg/cc) to standardize the grayscale values and maintain consistency between runs. Three-dimensional (3D) microstructural image data were reconstructed using NRecon software (SkyScan), and morphometric parameters were calculated using the SkyScan CT Analyzer (CTAn) software for trabecular and cortical bone in the femur. To analyze morphometric parameters of trabecular bone, the volume of interest (VOI) started at 0.17 mm under the growth plate of the femur extending 2 mm toward the diaphysis (2 mm in height) comprising trabecular bone and the marrow cavity [33]. To analyze morphometric parameters of cortical bone, the VOI started at the proximal end of the trabecular VOI extending 2 mm toward the mid shaft (2 mm in height) comprising only the cortical shell. An upper threshold of 600 and lower threshold of 0 were used to delineate each pixel as bone or not. Trabecular bone parameters (bone volume fraction [BV/TV, %], trabecular thickness [Tb.Th, μm], number [Tb.N, 1/mm], spacing [Tb.Sp, mm], and bone mineral density [BMD, mg/cc]), and cortical bone parameters (cortical area [Ct.Ar, mm^2], marrow area [Ma.Ar, mm^2], percent cortical area [Ct.Ar/Tt.Ar, %], and cortical thickness [Ct.Th, mm]) were measured according to guidelines for assessing bone microstructure in rodents using micro-CT [34].

2.6. Statistics

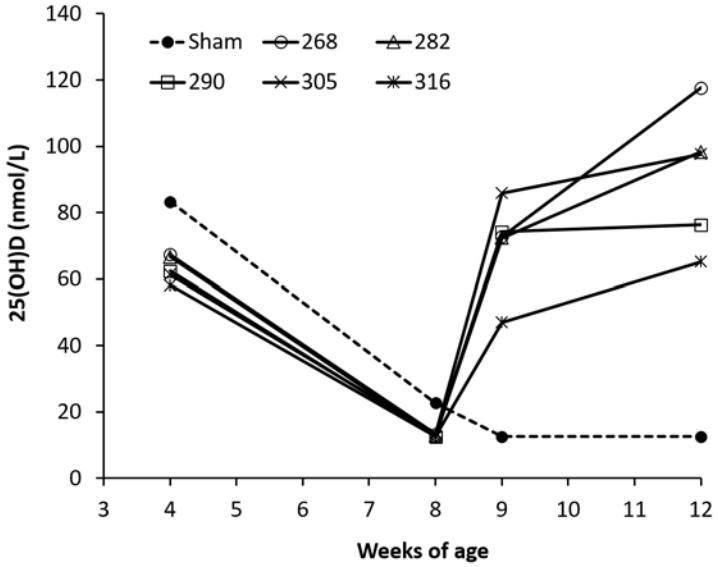
The results are presented as mean values ± standard deviation (SD). The Mann–Whitney U test was used to compare serum 25(OH)D level, serum $1,25(OH)_2D$ level, results of real-time RT-PCR, and results from micro CT measurements between each UV irradiation group and the control group. For the 25(OH)D and $1,25(OH)_2D$ level, an ANOVA with Tukey post hoc analyses was used for multiple comparisons among all wavelengths. All statistical analyses

were performed using SPSS statistics version 23 (IBM Corp. Armonk, NY). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of UV irradiation on serum 25(OH)D levels

From 4 to 12 weeks of age, mice were fed with the same vitamin D-deficient diet. To confirm the 25(OH)D deficient status of the mice at the time of pre-UV irradiation, we examined serum 25(OH)D levels at 4 weeks (pre-vitamin D deficient diet) and 8 weeks of age (initiation of UV irradiation). As indicated in Figure 4, serum 25(OH)D levels decreased to a deficiency level (< 25 nmol/L) by 8 weeks of age in all groups, and there was a significant difference in 25(OH)D levels between mice at 4 and 8 weeks of age ($P < 0.001$, all groups). Since the minimum detection limit of serum 25(OH)D was 12.5 nmol/L, we indicated 12.5 nmol/L for values which were less than the limit. At 9 weeks of age (1 week irradiation), serum 25(OH)D levels had increased rapidly in all UV irradiation groups ($P < 0.001$, all groups). Serum 25(OH)D levels continued to increase gradually or were maintained at the same level from 9 to 12 weeks of age, which was equal to vitamin D normal (25nm/L-90nm/L) or sufficiency (90nm/L<) levels in all UV irradiation groups at 12 weeks of age (Fig.4, Table 1). In contrast, control group mice subjected to incandescent light exhibited low 25(OH)D levels at 9 and 12 weeks of age.



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262 **Figure 4 Serum 25(OH)D levels.** Serum levels of 25(OH)D were determined at 4 weeks of
263 age (initiation of vitamin D deficient diet), 8 weeks of age (initiation of irradiation), 9 weeks
264 of age (1 week irradiation), and 12 weeks of age (4 weeks irradiation). Serum levels were
265 analyzed for 7 mice in each group.

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267

Table 1 Serum 25(OH)D_3 levels at various wavelength

	4 weeks of age	8 weeks of age	9 weeks of age		12 weeks of age	
wavelength	25(OH)D (SD; nmol/L)	25(OH)D (SD; nmol/L)	25(OH)D (SD; nmol/L)	*P* value	25(OH)D (SD; nmol/L)	*P* value
268 nm	67.4 (8.6)	13.3 (2.0)	72.8 (8.7)	< 0.001	117.7 (40.4)	< 0.001
282 nm	66.7 (12.3)	12.8 (0.9)	72.4 (6.9)	< 0.001	98.4 (28.2)	< 0.001
290 nm	62.4 (7.9)	N.D.	74.2 (12.9)	< 0.001	76.3 (26.1)	< 0.001
305 nm	61.3 (5.4)	13.2 (1.9)	85.9 (8.5)	< 0.001	97.7 (35.6)	< 0.001
316 nm	58.1 (11.9)	N.D.	47.1 (13.8)	< 0.001	65.3 (13.6)	< 0.001
Sham	83.3 (7.0)	22.7 (4.5)	N.D.		N.D.	

Statistical analyses was performed using by Mann-Whitney U test between each data and the value of sham group. N.D.; not detectable (<25nM/L).

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270 ***3.2. Effects of UV irradiation on serum 1,25(OH)$_2$D levels***

271 As indicated in Figure 5, serum 1,25(OH)$_2$D levels significantly decreased from 4 to 8 weeks

of age ($P < 0.001$, all groups), in parallel with serum 25(OH)D levels. UV irradiation did not increase serum $1,25(OH)_2D$ levels at 9 weeks of age (1 week irradiation), which exhibited different behavior from serum 25(OH)D levels (Fig.4). However, serum $1,25(OH)_2D$ levels in the UV irradiation groups at 12 weeks of age increased gradually or were maintained at the same levels as those at 9 weeks of age. In contrast, those in the control group continued to decrease from 9 to 12 weeks of age. Serum $1,25(OH)_2D$ levels in 268nm, 282nm, and 305nm of UV irradiation groups were significantly higher than those in the control group ($P<0.027$, $P<0.003$, and $P<0.011$, respectively) (Table 2).

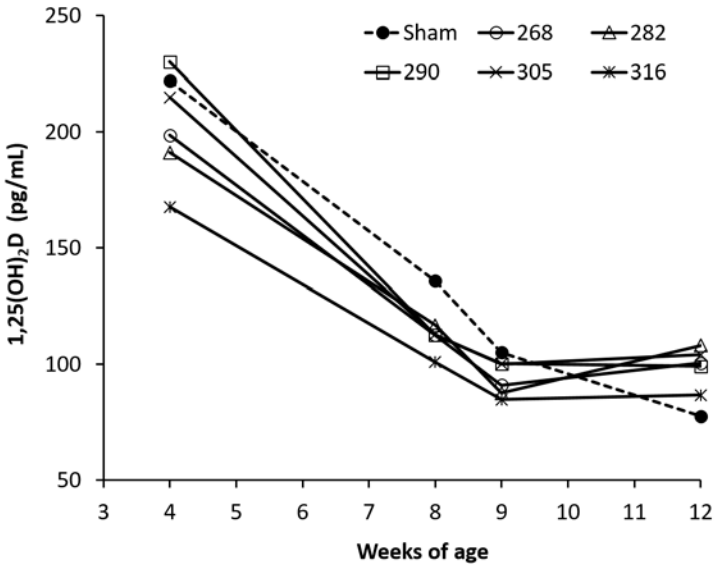

Figure 5 Serum $1,25(OH)_2D$ levels. Serum levels of $1,25(OH)_2D$ were determined at 4 weeks of age (initiation of vitamin D deficient diet), 8 weeks of age (initiation of irradiation), 9 weeks of age (1 week irradiation), and 12 weeks of age (4 weeks irradiation). Serum levels were analyzed for 7 mice in each group.

Table 2 Serum $1,25(OH)_2D_3$ levels at various wavelength

	4 weeks of age	8 weeks of age	9 weeks of age		12 weeks of age	
wavelength	$1,25(OH)_2D$ (SD; pgl/mL)	$1,25(OH)_2D$ (SD; pg/mL)	$1,25(OH)_2D$ (SD; pg/mL)	*P* value	$1,25(OH)_2D$ (SD; pg/mL)	*P* value
268 nm	198.7 (19.6)	112.5 (22.6)	91.1 (19.0)	0.098	100.6 (17.3)	0.027
282 nm	191.1 (33.4)	116.9 (36.1)	87.5 (25.5)	0.126	108.1 (12.8)	0.003
290 nm	230.1 (42.3)	112.6 (45.0)	100.2 (26.2)	0.328	99.1 (22.0)	0.069
305 nm	214.9 (42.0)	112.9 (40.0)	100.2 (46.7)	0.930	104.2 (15.8)	0.011
316 nm	167.7 (35.1)	101.0 (27.5)	84.8 (32.3)	0.375	86.8 (22.8)	0.328
Sham	222.0 (46.5)	135.8 (26.5)	105.1 (22.0)		77.6 (19.6)	

Statistical analyses was performed using by Mann-Whitney U test between each data and the value of sham group.

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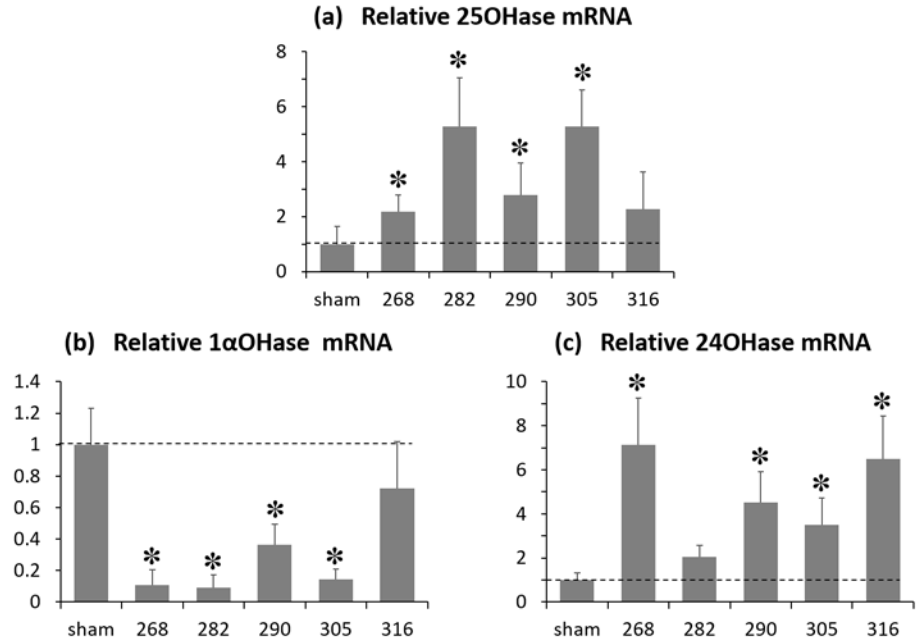
290 ***3.3. Difference in effects between UV-B and UV-A (316nm) wavelength on vitamin D levels***

291 $1,25(OH)_2D$, which is the active form of vitamin D, is the final product of the vitamin D
292 metabolic pathway. The magnitude of the normal concentration of serum $1,25(OH)_2D$ (pg/ml)
293 is much lower than that of 25(OH)D (ng/ml). In addition, $1,25(OH)_2D$ levels are delicately
294 controlled by internal maintenance of homeostasis including the concentrations of calcium
295 and $1,25(OH)_2D$ [35], suggesting that 25(OH)D might be more appropriate as an index to
296 evaluate the short-term effects of UV irradiation on vitamin D metabolism than $1,25(OH)_2D$.
297 As indicated in Table 1, UV irradiation in all wavelengths (including UV-A, UV-B and
298 UV-C) had significant effects on the increase of vitamin D levels. Serum 25(OH)D levels in
299 the 316 nm (UV-A) irradiation group increased more slowly compared with mice in UV-B or
300 UV-C irradiation groups. However, the serum levels in UV-A increased within the normal
301 range of vitamin D levels at 9 or 12 weeks of age the same as those in UV-B or UV-C groups
302 (Figure 4). The results of multiple comparison analysis among all wavelengths showed that
303 316 nm UV irradiation had a significantly lower effect on vitamin D levels than other
304 wavelengths at 9 weeks of age (= irradiation 1 week) ($P < 0.05$), although there was no
305 significant difference at 12 weeks of age (= irradiation 4 weeks).

306

307 ***3.4. Levels of mRNA expression responsible for vitamin D metabolism***

We investigated levels of mRNA expression for vitamin D 25-hydroxylase (Cyp27a1), 25 hydroxyvitamin D-1-alpha hydroxylase (Cyp27b1), and 1,25-dihydroxyvitamin D 24-hydroxylase (Cyp24a1) by real-time RT-PCR. The mRNA levels of vitamin D 25-hydroxylase, which is responsible for the conversion of vitamin D into the stored form, 25(OH)D, were significantly higher in all UV-B or UV-C irradiation groups compared with the control group (Figure 6). The results suggested that more vitamin D converted from 7-dehydrocholesterol at the irradiated skin by UV was transported to the liver, where up-regulated 25-hydroxylase might convert vitamin D to 25(OH)D. The mRNA levels of 25 hydroxyvitamin D-1-alpha hydroxylase, which is responsible for the conversion of 25(OH)D into the active form $1,25(OH)_2D$ in the kidney, were significantly lower in all UV-B or UV-C irradiation groups compared with the control group. In contrast, the mRNA levels of 1,25-dihydroxyvitamin D 24-hydroxylase, which is responsible for the conversion of active $1,25(OH)_2D$ into the inactive form, were significantly higher in most of the UV irradiation groups than those in the control group (Figure 6). This reciprocal regulation of the renal 25 hydroxyvitamin D-1-alpha hydroxylase and 1,25-dihydroxyvitamin D 24-hydroxylase might be due to the homeostatic response of $1,25(OH)_2D$ and calcium on the regulation of vitamin D hormone levels. This homeostatic regulation could explain why UV irradiation did not significantly increase serum $1,25(OH)_2D$ levels at either 9 weeks or 12 weeks of age, while serum 25(OH)D levels did so (Figure 4,5).



328

329 **Figure 6 Levels of mRNA expression for vitamin D metabolism.** Relative expression
330 levels in each irradiated group are expressed with reference to that in the control group as 1.0.
331 Levels of all target mRNA were normalized with those of Gapdh mRNA. (a) Relative
332 25OHase mRNAs were significantly higher in all UV-B or UV-C irradiation groups
333 compared with the control group. (b) Relative 1αOHase mRNAs were significantly lower in
334 all UV-B or UV-C irradiation groups compared with the control group. (c) Relative 24OHase
335 mRNAs were significantly higher in most of the UV irradiation groups compared with the
336 control group. *P<0.05 as determined by Mann-Whitney U test v.s. control (vitamin D
337 deficient dietary, incandescent light) group.

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340 ***3.5. Analyses of bone morphology with micro-CT measurement***

341 As preliminary experiments for micro-CT analyses, we evaluated the reliability of micro-CT
342 measurements using 10 femur samples of mice. Intra-class reliabilities of Tb.BV/TV,

343 Tb.BMD, and Ct.Th as the main bone volume parameters were calculated using Spearman's
344 correlation coefficient (Table 3).

Table 3 Intra-class reliabilities of micro computed tomography

group	ICC-1†	ICC-2§
Tb.BV/TV	0.987	0.979
Tb.BMD	0.971	0.959
Ct.Th	0.838	0.845

† Intra-class reliability between two measurements for the same reconstructive microstructural image using CT Analyzer software. §Intra-class reliability between two reconstructive microstructural images for the same sample. Tb.BV/TV, trabecular percent bone volume fraction; Tb.BMD, trabecular bone mineral density; Ct.Th, cortical thickness.

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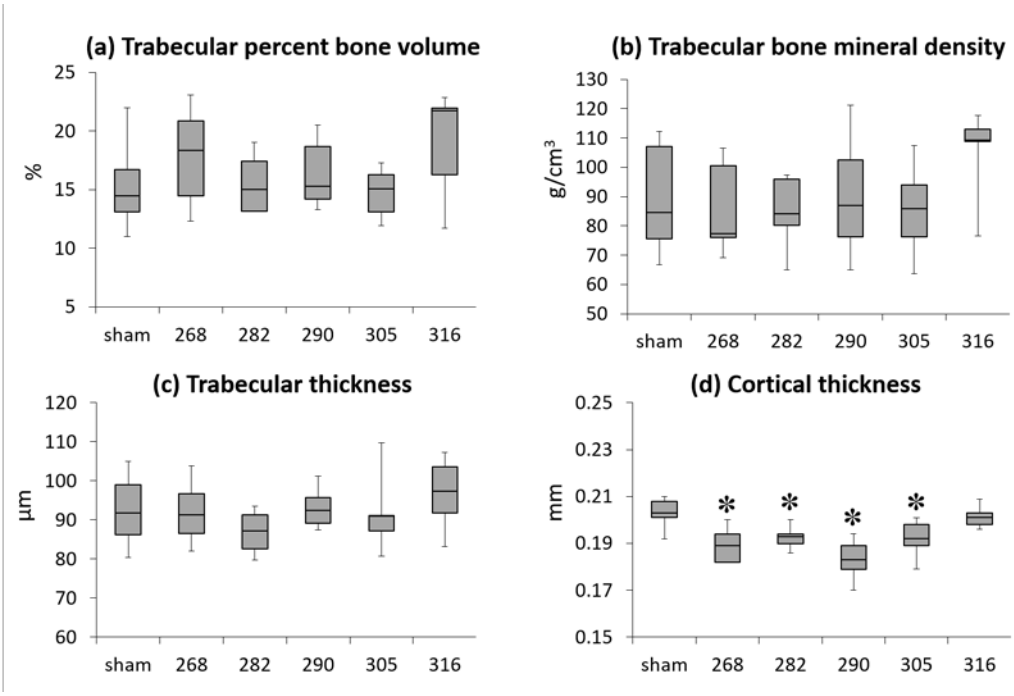
347 After confirmation of the reliabilities, we examined each bone parameter at 12 weeks of age
348 (post-4 weeks UV irradiation) using a micro-CT for the distal femur trabecular region and the
349 mid-distal femur cortical region. No significant improvements in any trabecular or cortical
350 bone parameters were noted in the UV irradiation groups compared with those in the control
351 group. As for analyses of trabecular bone, all of the values in each parameter were similar in
352 all of the groups (Table 4). However, values of the main trabecular bone volume parameters
353 (Tb.BV/TV, Tb.BMD) in the 316 nm UV irradiation group tended to be higher than those in
354 the control and other UV-B or UV-C irradiation groups (Figure 7). As for analyses of cortical
355 bone, values of parameters for cortical bone volume (Ct.Ar, Ct.Ar/Tt.Ar, and Ct.Th) in UV
356 irradiation groups were lower than those in the control group, except for the 316 nm UV
357 irradiation group (Table 4).

358



359

360 **Figure 7 Results of main bone parameters with micro-CT analyses.** (a); trabecular percent
361 bone volume [Tb.BV/TV], (b); trabecular bone mineral density [Tb.BMD], (c); trabecular
362 thickness [Tb.Th], (d); cortical thickness [Ct.Th]. Values of each bone parameter were
363 determined with micro-CT at 12 weeks of age (4 weeks irradiation). Graphs are depicted as
364 box-and whisker plots. A rectangle is drawn to represent the second and third quartiles with a
365 horizontal line inside to indicate the median value. The lower and upper quartiles are shown
366 as vertical lines on either side of the rectangle. *P<0.05 as determined by Mann-Whitney U
367 test v.s. control (vitamin D deficient dietary, incandescent light) group.

368

369

Table 4 Micro computed tomography analyses for morphological parameters of trabecular and cortical bone at 12 weeks of age

Parameters	268 nm	282 nm	290 nm	305 nm	316 nm	Control
Tb. BV/TV (%)	17.8 ± 4.4	15.6 ± 2.6	16.3 ± 3.1	14.7 ± 2.1	18.9 ± 4.8	15.3 ± 3.5
Tb. Th (μm)	91.3 ± 8.8	87.1 ± 5.8	92.5 ± 5.5	91.0 ± 9.1	97.4 ± 9.8	91.8 ± 9.2
Tb. N (1/mm)	1.9 ± 0.4	1.8 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	1.7 ± 0.3	1.7 ± 0.3
Tb. Sp (mm)	0.37 ± 0.08	0.36 ± 0.05	0.45 ± 0.12	0.44 ± 0.11	0.36 ± 0.05	0.36 ± 0.05
Tb. BMD (mg/cm^3)	86 ± 17	85 ± 13	90 ± 21	85 ± 15	89 ± 17	89 ± 17
Ct. Ar (mm^2)	1.01 ± 0.05	0.95 ± 0.02*	0.92 ± 0.04*	0.95 ± 0.06*	1.02 ± 0.05	1.00 ± 0.04
Ma. Ar (mm^2)	1.06 ± 0.07	1.13 ± 0.07	1.19 ± 0.05*	1.13 ± 0.06	1.15 ± 0.07	1.06 ± 0.06
Ct.Ar / Tt.Ar (%)	48.6 ± 2.6	45.7 ± 1.3*	43.7 ± 1.8*	45.6 ± 1.0*	47.2 ± 1.1	48.5 ± 1.6
Ct. Th (mm)	0.189 ± 0.009*	0.192 ± 0.005*	0.183 ± 0.009*	0.192 ± 0.008*	0.201 ± 0.006	0.203 ± 0.007

Statistical analyses were performed using by Mann-Whitney U test. ✻ $P < 0.05$; v.s. control group. Mice of each group had 8 weeks of vitamin D deficient dietary (4 weeks of age to 12 weeks of age) and 4 weeks of ultraviolet irradiation (8 weeks of age to 12 weeks of age) or an incandescent light (control group). BV, bone volume; TV, tissue volume; Tb.BV/TV, trabecular percent bone volume fraction; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.BMD, trabecular bone mineral density; Ct.Ar, cortical area; Ma.Ar, marrow area; Tt.Ar; tissue area; Ct.Ar/Tt.Ar, percent cortical area; Ct.Th, cortical thickness.

370 ## 4. Discussion

371 In this study, we demonstrated favorable effects of UV irradiation on converted vitamin D
372 levels as well as the differential effects of various UV wavelengths in an *in vivo* mouse model
373 using a new LED technology that can generate narrow-range wavelengths of UV compared
374 with existing incandescent or fluorescent lamps. Although some previous *in vivo* experiments
375 showed that UV irradiation increases vitamin D levels, UV sources in those studies were
376 incandescent or fluorescent UV lamps with comparatively broad-range wavelengths [27, 30,
377 36, 37]. Therefore, detailed examinations *in v*ivo are required to analyze the different effects
378 of narrow-range wavelengths with UV irradiation on the vitamin D supply. *In vitro*,
379 MacLaughlin et al. [26] and the CIE (Comite International de I' Eclairage) report [38]
380 provided the action spectrum for the UV-induced previtamin D_3 in human skin. MacLaughlin
381 et al. [26] concluded, based on the results of their analyses, that the peak of the action

spectrum was 297 nm. They also reported that the effectiveness of previtamin D_3 production at 310 nm decreased to 25% of that at 297 nm, with no production of previtamin D_3 observed at 320 nm. The CIE action spectrum report [38], in which the figure of the action spectrum reported by MacLaughlin et al. [26] was taken as the starting point, showed that the peak of the action spectrum was 298 nm, and previtamin D_3 was produced almost entirely in the UV-B band (280-315nm) with only about 3–4% of total production in the UVA wavelengths. The CIE report [38] also indicated that the previtamin D_3 production at 316 nm was 0.02 times that at 298 nm.

Our study did not determine the action spectrum for the production of previtamin D_3 directly, but evaluated serum levels of 25(OH)D and 1,25$(OH)_2$D which is the metabolite of previtamin D_3 in the vitamin D metabolic pathway following LED-UV irradiation. Remarkable findings of the present study were that at the short-range wavelength of 316 nm, UV-A could increase the serum 25(OH)D levels to more than half of those at other UV-B or UV-C wavelengths less than 305 nm at 9 weeks of age (1 week of irradiation), and to the same as those of UV-B or UV-C at 12 weeks of age (4 weeks of irradiation). The discrepancy between the present study and MacLaughlin et al's one. [26] and the CIE report [38] is possibly explained as suggested by Norval et al. [39]. MacLaughlin's report did not provide the information for dose at each wavelength, site and age for prepared human skin, or the temperature at irradiation. The second reason for the upregulation of serum 25(OH)D levels by 316 nm may be that the narrow-range UV-LED used in the present study had a 20-40 nm width at the bottom, which would affect the conversion to previtamin D_3. For example, 316 nm of LED-UV provided a small amount of 300 nm of wavelength, which would influence the conversion to previtamin D_3 at the skin. The third reason is likely related to the complicated metabolic pathway of vitamin D. The action spectrum is not a simple

406 representation of the single forward reaction of 7-DHC to previtamin D_3. Back reaction from
407 previtamin D_3 to 7-DHC or the formation and back reaction of tachysterol or lumisterol,
408 which are the inactive isomers, will have effects on the production of previtamin D_3 and
409 25(OH)D [39-43].
410 Future endpoints of the present study are to increase the serum levels of activated vitamin D,
411 subsequently to raise the bone mineral density (BMD), and decrease the numbers of elderly
412 patients with fractures due to osteoporosis, which would be expected to improve their quality
413 of life and healthy life expectancy. A previous study investigated the usefulness of sunlamps
414 emitting 0.5% (or 1.4%) of UVB and 99.5% (or 98.6%) of UVA to increase human serum
415 25(OH)D levels [44]. The results indicated that broad-range sunlamps increased serum levels
416 of 25(OH)D, resulting, however, in side effects such as erythema or polymorphic light
417 eruption. They did not investigate the bone morphology including BMD after irradiation with
418 sunlamps. Finally, the authors did not recommend the use of a sunbed as the vitamin D source.
419 Another study analyzed the effects of UV exposures from two artificial sources (one emitting
420 mainly UV-A, and the other primarily UV-B, mimicking winter and summer noon sunlight,
421 respectively) on the serum 25(OH)D concentration [45]. The major findings of their study
422 were that both lamps with main exposure of UV-A and UV-B could increase the serum levels
423 of 25(OH)D, and that there was a high variability in 25(OH)D and its response to UV
424 irradiation from person to person. To examine the wavelength-dependent differences in
425 effects on serum levels of 25(OH)D, irradiation with short-range wavelength lamps would be
426 desirable; moreover, side effects were also evaluable using LED-UV in the present study.
427 Other benefits using LED-UV with short-range wavelength include the non-necessity of
428 prolonged sunbathing, particularly in winter. Furthermore, the LED-UV device used in the
429 present study was small, portable and easy to use in a variety of clinical settings such as UV

irradiation only in unilateral upper limb. A 2 × 4 cm dorsal patch of clean-shaven skin of mice was approximately converted to 10 % of body surface area considering the mean body weights at 8 and 12 weeks of age were 17.9 ± 1.03 g and 19.5 ± 1.28 g (data was not shown) [46]. This percentage of body surface area is almost equal to surface area of unilateral upper limb in human [47].

However, it is essential to examine skin damage by UV irradiation and the minimum effective dose for vitamin D supplementation. We applied the dose of 1 kJ/m^2 twice a week in this study which was considered as suberythemal dose and confirmed to increase serum 25(OH)D levels [30]. However, in this study, the potential risk for skin inflammation could be higher even if the irradiation dose is equal to that of the previous study, because we used narrow range UV wavelengths. Moreover, the minimum effective dose of each wavelength for vitamin D supplementation is unclear. As a future plan, we will evaluate influences of various lower doses and cycles of UV irradiation on skin damage using by only 316 nm of wavelength as the most harmless wavelength in our modules.

One of the limitations of the present study was that LED-UV irradiation could not be demonstrated to improve bone morphology with micro-CT measurement at the time of 4 weeks' treatment. Vitamin D deficient status with rapid increase of serum 25(OH)D levels with LED-UV irradiation might be associated with the results of micro-CT. Vitamin D promotes the intestinal absorption of calcium and phosphorus, necessary for bone mineralization [48]. In the case of calcium shortage with high vitamin D levels, bone resorption and decreased bone mineralization are required to maintain normal calcium levels [49, 50]. In the present study, the reaction of calcium elution from bone might have been more significant than intestinal absorption of calcium by rapid increases in vitamin D with hypocalcemia status at 4 weeks of a vitamin D-deficient diet before UV irradiation.

Longer-term evaluation with standard levels of calcium status may provide more favorable results by micro-CT measurement. The second limitation is the adequacy of the mice vitamin D deficient model used in this study. Young-adult mice were fed a vitamin D-deficient diet for 8 weeks in advance of UV irradiation. A previous study reported that osteoporosis may occur in adult rats with a vitamin D-deficient diet and adequate calcium intake for 6 months [51]. Bone morphometric changes might not have occurred in mice because of the short duration of the vitamin D-deficient diet used in this study.

Another limitation was the lack of any comparison with the effects of vitamin D administration, which has been established to be one of the most effective treatments for osteoporosis [5-11], on serum levels of vitamin D. Every patient with osteoporosis should take vitamin D supplementation individually; however, LED-UV irradiation could be provided as repeated therapy for different patients with the same device, contributing to a reduction in the associated medical costs. Patients with malabsorption could be treated with LED-UV.

In conclusion, UV irradiation at narrow-range wavelengths provided by a UV-LED lamp is effective for increasing vitamin D levels even at 316 nm, which had previously been considered as a non-effective wavelength for previtamin D_3 production. This study provides essential information on the future development of a therapeutic UV-LED device for osteoporosis. Considering that many developed countries face an increasingly super-aging society, and increasing numbers of patients will be burdened by osteoporosis and its complications, irradiation with UV-LED device has promise to become a new and epoch-defining treatment possibly with decreased side effects.

Acknowledgments

We would like to express our gratitude to Nikkiso Co ltd., Tokyo, Japan for their technical cooperation in developing the therapeutic UV-LED instrument. We thank MS. Eri Ishihara for her secretarial assistance.

Conflict of interest and Source of Funding

The authors have no conflicts of interest to declare. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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