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

Daigo Morita, M.D. [a], Yoshihiro Nishida, M.D., Ph.D. [a,*], Yoshitoshi Higuchi, M.D. [a], Taisuke Seki, M.D. [a], Kunihiro Ikuta, M.D. [a], Hideki Asano [b], Naoki Ishiguro, M.D., Ph.D. [a]

[a] Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya City 466-8550, Japan
[b] Development Department, NIKKISO GIKEN Co., Ltd., 4-20-3, Ebisu, Shibuya-ku, Tokyo 150-6022, Japan

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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$(OH)_2$D 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 (268 nm–305 nm), 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.

1. Introduction

Osteoporosis is characterized by low bone mass, progressive bone loss, and microarchitectural 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 >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$(OH)_2$D 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$(OH)_2$D 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$(OH)_2$D led to an up-regulation 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

* Corresponding author.
E-mail addresses: daigood0301@yahoo.co.jp (D. Morita), ynishida@med.nagoya-u.ac.jp (Y. Nishida), rdggd215@yahoo.co.jp (Y. Higuchi), taiseki@med.nagoya-u.ac.jp (T. Seki), k-ikuta@med.nagoya-u.ac.jp (K. Ikuta), hideki.asano@nikkiso.co.jp (H. Asano), n-ishi@med.nagoya-u.ac.jp (N. Ishiguro).

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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 >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 Fig. 1.

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 evaluate effective UV wavelength in detail, we planned to use four UVB wavelengths and one UVC wavelength in this study; the shortest and longest wavelength of UVB (280 and 315 nm), and the two wavelengths to divide UVB range equally (292 and 304 nm), and UVC wavelength (265 nm). During manufacturing by Nikkiso Co ltd, wavelengths emitted by LED modules were adjusted to 282 nm, 290 nm, 305 nm (UVB), 268 nm (UVC) and 316 nm (the short wavelength end of UVA). 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).

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

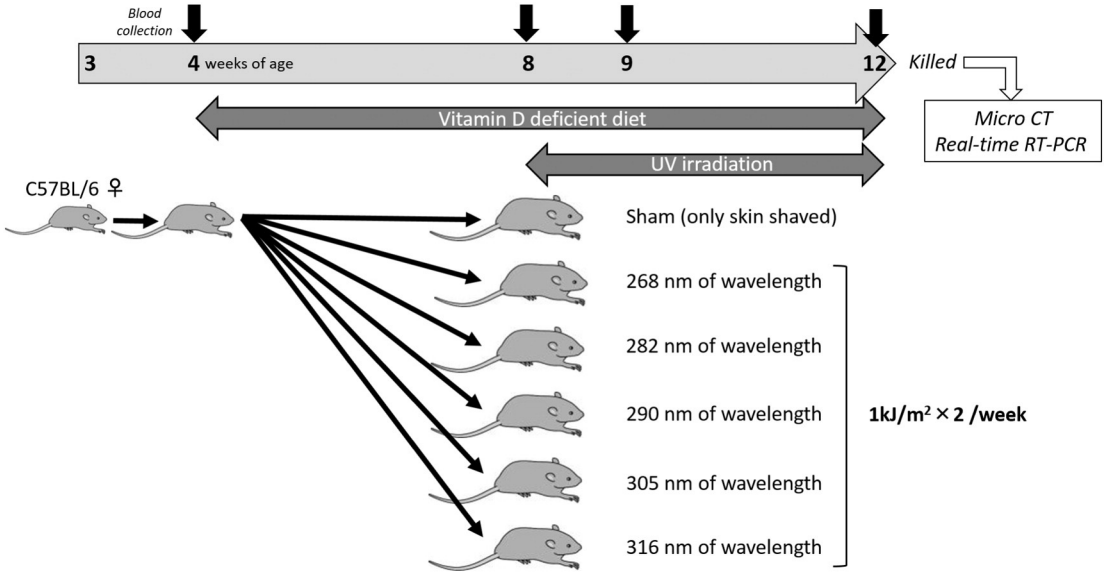

Fig. 1. The experimental protocol. Inbred C57BL/6 female mice at 4 weeks of age were fed a vitamin D deficient-diet until the termination of this study (12 weeks of age). At 8 weeks of age, the mice were divided into a control group (skin was shaved, without irradiation) and five UV irradiation groups of each wavelength (268 nm, 282 nm, 290 nm, 305 nm, and 316 nm). The mice were irradiated with UV irradiation regimens (1 kJ/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.

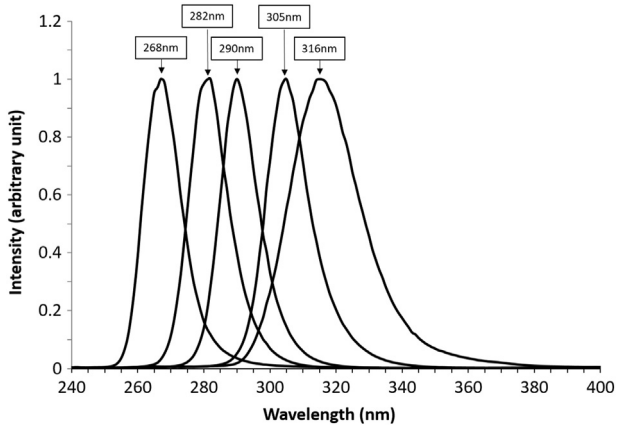

Fig. 2. Relative spectral irradiance of prepared LED modules. The wave spectrums and intensity of each LED module (268, 282, 290, 305, and 316 nm) were measured using a UV radiometer under the same conditions.

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. UV irradiation dose was controlled to 1 kJ/m^2 twice a week, which was considered as suberythemal dose, and reported to increase serum 25(OH)D levels in a previous study [30]. Radiation irradiance was set at an identical to 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 s 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 (185 s) 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.

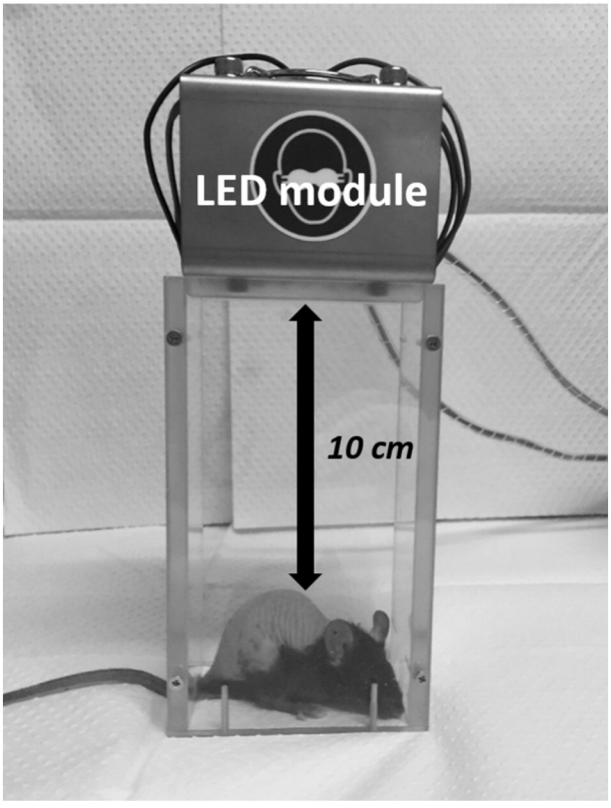

Fig. 3. Scene of mouse irradiation with LED module. The LED lamps were positioned on an acrylic box 10 cm above the dorsal patch where the skin was shaved for irradiation.

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).

Total RNA was isolated from kidney and liver of each mouse using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol of the supplier. Reverse transcribed cDNA was subjected to real-time RT-PCR using a LightCycler 480 (Roche Diagnostics, Mannheim, Germany), with 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany), using 0.5 μM of the sense and antisense specific primers. The conventional amplification program was applied; preincubation step for denaturation of the template cDNA (10 min, 95 °C), followed by 45 cycles of a denaturation step (10 s, 95 °C), an annealing step (10 s, 60 °C), and an extension step (10 s, 72 °C). Every run included a negative control without cDNA template. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis on the LightCycler 480 and also a 2% agarose/TAE gel electrophoresis, to measure Tm and amplicon size, respectively. To allow relative quantification after PCR, real-time efficiencies were calculated from the given slopes in LightCycler 480 software (Roche Diagnostics, Mannheim, Germany) using serial dilutions. The relative levels of mRNAs in a sample were expressed after normalization with those of glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The primer pairs used for Gapdh, Cyp27a1, Cyp27b1, and Cyp24a1 were designed according to the report by Satué et al. [32].

2.5. Analyses with Micro-Computed Tomography (CT)

The influence of altered 25(OH)D and 1,25(OH)$_2$D with UV irradiation on trabecular and cortical microarchitectures of treated mice was assessed using the distal femur metaphysis at 12 weeks of age. Right femurs fixed in 70% ethanol were subjected to scanning with a high-resolution micro-CT scanner using specific software (SkyScan 1176, SkyScan, Kontich, Belgium). Briefly, each scan was performed with a source voltage of 50 kV, current of 500 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 s. Scans also included phantom bones for analysis of bone mineral

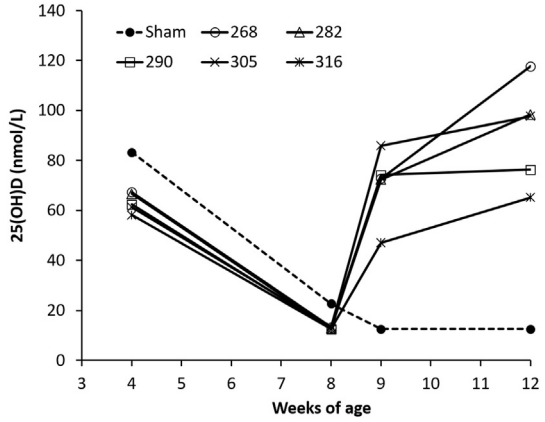

Fig. 4. Serum 25(OH)D levels. Serum levels of 25(OH)D were determined at 4 weeks of age (initiation of vitamin D deficient diet), 8 weeks of age (initiation of irradiation), 9 weeks of age (1 week irradiation), and 12 weeks of age (4 weeks irradiation). Serum levels were analyzed for 7 mice in each group.

density (250 mg/cm^3 and 750 mg/cm^3) 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/cm^3]), 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$(OH)_2$D 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$(OH)_2$D 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 Fig. 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 (25 nmol/L-90 nmol/L) or sufficiency (> 90 nmol/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.

3.2. Effects of UV Irradiation on Serum 1,25$(OH)_2$D Levels

As indicated in Fig. 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$(OH)_2$D 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$(OH)_2$D 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$(OH)_2$D levels in 268 nm, 282 nm, and 305 nm 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).

3.3. Difference in Effects Between UV-B and UV-A (316 nm) Wavelength on Vitamin D Levels

1,25$(OH)_2$D, which is the active form of vitamin D, is the final product of the vitamin D metabolic pathway. The magnitude of the normal concentration of serum 1,25$(OH)_2$D (pg/ml) is much lower than that of 25(OH)D (ng/ml). In addition, 1,25$(OH)_2$D levels are delicately controlled by internal maintenance of homeostasis including the concentrations of calcium and 1,25$(OH)_2$D [35], suggesting that 25(OH)D might be more appropriate as an index to evaluate the short-term effects of UV irradiation on vitamin D metabolism than 1,25$(OH)_2$D. As indicated in Table 1, UV irradiation in all wavelengths (including UV-A, UV-B and UV-C) had significant effects on the increase of vitamin D levels. Serum 25(OH)D levels in the 316 nm (UV-A) irradiation group increased more slowly compared with mice in UV-B or UV-C irradiation

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

Wavelength	4 weeks of age	8 weeks of age	9 weeks of age	12 weeks of age		
	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 (<25 nmol/L).

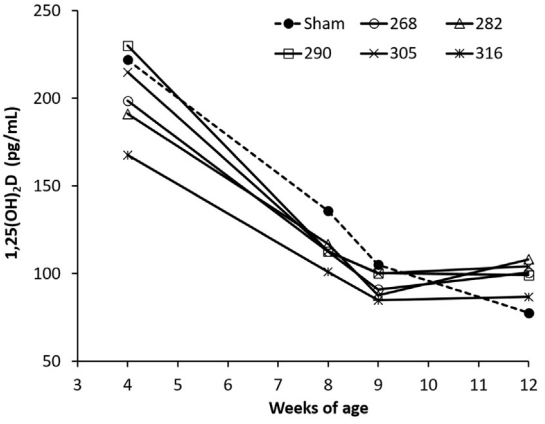

Fig. 5. Serum 1,25(OH)$_2$D levels. Serum levels of 1,25(OH)$_2$D were determined at 4 weeks of age (initiation of vitamin D deficient diet), 8 weeks of age (initiation of irradiation), 9 weeks of age (1 week irradiation), and 12 weeks of age (4 weeks irradiation). Serum levels were analyzed for 7 mice in each group.

groups. However, the serum levels in UV-A increased within the normal range of vitamin D levels at 9 or 12 weeks of age the same as those in UV-B or UV-C groups (Fig. 4). The results of multiple comparison analysis among all wavelengths showed that 316 nm UV irradiation had a significantly lower effect on vitamin D levels than other wavelengths at 9 weeks of age (= irradiation 1 week) ($P < 0.05$), although there was no significant difference at 12 weeks of age (= irradiation 4 weeks).

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 (Fig. 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)$_2$D 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)$_2$D into the inactive form, were significantly higher in most of the UV irradiation groups than those in the control group (Fig. 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)$_2$D 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)$_2$D levels at either 9 weeks or 12 weeks of age, while serum 25(OH)D levels did so (Figs. 4, 5).

3.5. Analyses of Bone Morphology With Micro-CT Measurement

As preliminary experiments for micro-CT analyses, we evaluated the reliability of micro-CT measurements using 10 femur samples of mice. Intra-class reliabilities of Tb.BV/TV, Tb.BMD, and Ct.Th as the main bone volume parameters were calculated using Spearman's correlation coefficient (Table 3).

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

4. Discussion

In this study, we demonstrated favorable effects of UV irradiation on converted vitamin D levels as well as the differential effects of various UV wavelengths in an *in vivo* mouse model using a new LED technology that can generate narrow-range wavelengths of UV compared with existing incandescent or fluorescent lamps. Although some previous *in vivo* experiments showed that UV irradiation increases vitamin D levels, UV sources in those studies were incandescent or fluorescent UV lamps with comparatively broad-range wavelengths [27,30,36,37]. Therefore, detailed examinations *in vivo* are required to analyze the different effects of narrow-range wavelengths with UV irradiation on the vitamin D supply. *In vitro*, MacLaughlin et al. [26] and the CIE (Comite International de I' Eclairage) report [38] provided the action spectrum for the UV-induced previtamin D_3 in human skin. MacLaughlin 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 (280-315 nm) 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

Table 2
Serum 1,25(OH)$_2$D$_3$ levels at various wavelength.

Wavelength	4 weeks of age	8 weeks of age	9 weeks of age	12 weeks of age		
	1,25(OH)$_2$D (SD; pg/mL)	1,25(OH)$_2$D (SD; pg/mL)	1,25(OH)$_2$D (SD; pg/mL)	*P* value	1,25(OH)$_2$D (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.

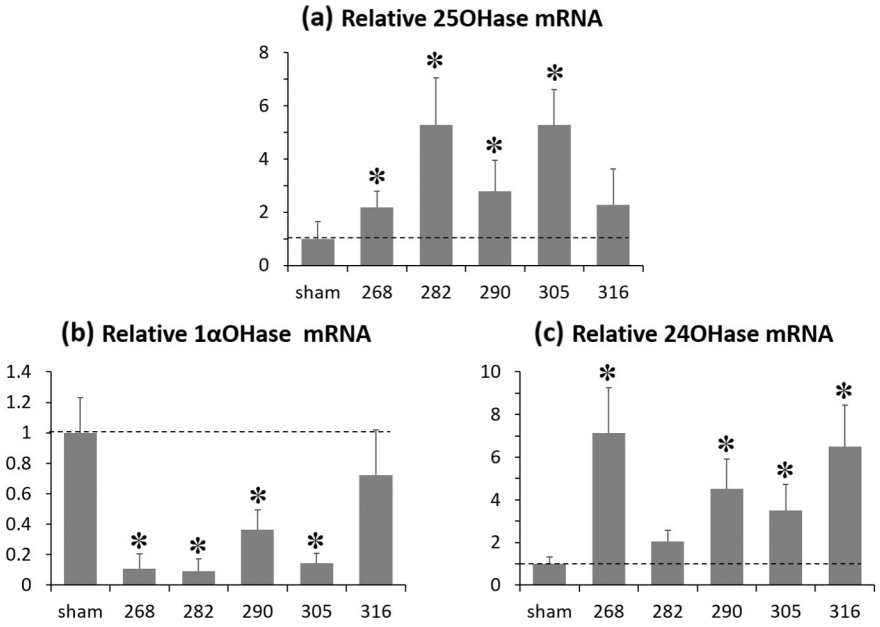

Fig. 6. Levels of mRNA expression for vitamin D metabolism. Relative expression levels in each irradiated group are expressed with reference to that in the control group as 1.0. Levels of all target mRNA were normalized with those of Gapdh mRNA. (a) Relative 25OHase mRNAs were significantly higher in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

$1,25(OH)_2D$ 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 < 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 representation of the single forward reaction of 7-DHC to previtamin D_3. Back reaction from previtamin D_3 to 7-DHC or the formation and back reaction of tachysterol or lumisterol, which are the inactive isomers, will have effects on the production of previtamin D_3 and 25(OH)D [39–43].

Future endpoints of the present study are to increase the serum levels of activated vitamin D, subsequently to raise the bone mineral density (BMD), and decrease the numbers of elderly patients with fractures due to osteoporosis, which would be expected to improve their quality of life and healthy life expectancy. A previous study investigated the usefulness of sunlamps emitting 0.5% (or 1.4%) of UVB and 99.5% (or 98.6%) of UVA to increase human serum 25(OH)D levels [44]. The results indicated that broad-range sunlamps increased serum levels of 25(OH)D, resulting, however, in side effects such as erythema or polymorphic light eruption. They did not investigate the bone morphology including BMD after irradiation with sunlamps. Finally, the authors did not recommend the use of a sunbed as the vitamin D source. Another study analyzed the effects of UV exposures from two artificial sources (one emitting mainly UV-A, and the other primarily UV-B, mimicking winter and summer noon sunlight, respectively) on the serum 25(OH)D concentration [45]. The major findings of their study were that both lamps with main exposure of UV-A and UV-B could increase the serum levels of 25(OH)D, and that there was a high variability in 25(OH)D and its response to UV irradiation from person to person. To examine the wavelength-dependent differences in effects on serum levels of 25(OH)D, irradiation with short-range wavelength lamps would be desirable; moreover, side effects were also evaluable using LED-UV in the present study. Other benefits using LED-UV with short-range wavelength include the non-necessity of prolonged sunbathing, particularly in winter. Furthermore, the LED-UV device used in the 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 used in the present study 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 [46]. This percentage of body surface area is almost equal to that of unilateral upper limb in human [47].

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

Table 3
Intra-class reliabilities of micro computed tomography.

Group	ICC-1[a]	ICC-2[b]
Tb.BV/TV	0.987	0.979
Tb.BMD	0.971	0.959
Ct.Th	0.838	0.845

Tb.BV/TV, trabecular percent bone volume fraction; Tb.BMD, trabecular bone mineral density; Ct.Th, cortical thickness.

[a] Intra-class reliability between two measurements for the same reconstructive microstructural image using CT Analyzer software.

[b] Intra-class reliability between two reconstructive microstructural images for the same sample.

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. 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.

* $P < 0.05$; vs. control group.

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. One of the other possible tools to evaluate bone morphology is histological analyses of bones after irradiation on a short-term studies, which should be examined in further experiments. 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.

The third 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.

Other limitation is that skin damage by UV irradiation should be analyzed in detail in addition to 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, 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. We should evaluate influences of various lower doses and cycles of UV irradiation on skin damage in addition to the benefit to bones.

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.

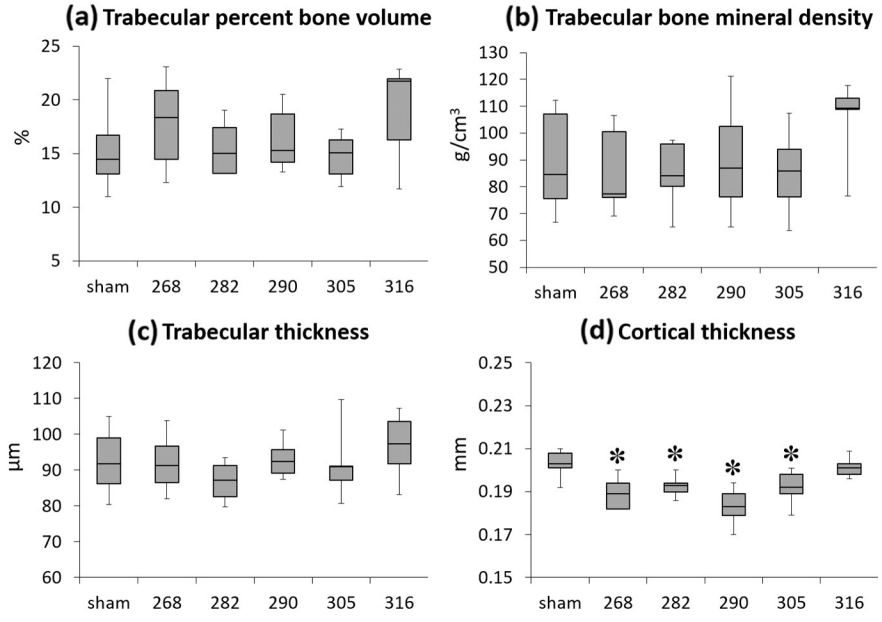

Fig. 7. Results of main bone parameters with micro-CT analyses. (a); trabecular percent bone volume [Tb.BV/TV], (b); trabecular bone mineral density [Tb.BMD], (c); trabecular thickness [Tb.Th], (d); cortical thickness [Ct.Th]. Values of each bone parameter were determined with micro-CT at 12 weeks of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

Conflict of Interest and Source of Funding

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References

[1] Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO study group. WHO technical report series 1994, 843.
[2] L.G. Raisz, Pathogenesis of osteoporosis: concepts, conflicts, and prospects, J. Clin. Invest. 115 (2005) 3318–3325.
[3] L.G. Raisz, C.L. Trummel, M.F. Holick, J.F. DeLuca, 1,25 Dihydroxyvitamin D3: a potent stimulator of bone resorption in tissue culture, Science 175 (1972) 768–769.
[4] T.A. Owen, M.S. Aronow, L.M. Barone, B. Bettencourt, G.S. Stein, J.B. Lian, Pleiotropic effect of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure and bone matrix competency in normal rat osteoblasts, Endocrinology 128 (1991) 1496–1504.
[5] P.A. Price, S.A. Baukol, 1,25 Dihydroxyvitamin D3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells, J. Biol. Chem. 255 (1980) 660–663.
[6] C.W. Prince, W.T. Butler, 1,25 Dihydroxyvitamin D3 regulates the biosynthesis of osteopontin, a bone derived cell attachment protein in clonal osteoblast-like osteosarcoma cells, Collagen Relat Res 7 (1987) 305–313.
[7] T. Shibata, A. Shira-Ishi, T. Sato, T. Masaki, A. Masuda, A. Hishiya, N. Ishikura, S. Higashi, Y. Uchida, M.O. Saito, M. Ito, E. Ogata, K. Watanabe, K. Ikeda, Vitamin D hormone inhibits osteoclastogenesis in vivo by decreasing the pool of osteoclast precursors in bone marrow, J. Bone Miner. Res. 17 (2002) 622–629.
[8] T. Matsumoto, C. Igarachi, Y. Taksushi, S. Harada, T. Kikuchi, H. Yamato, E. Ogata, Stimulation by 1,25 dihydroxyvitamin D3 of in vitro mineralization induced by osteoblast-like MCT3-E1 cells, Bone 12 (1991) 27–32.
[9] P. Lips, Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications, Endocr. Rev. 22 (2001) 477–501.
[10] T. Suda, Y. Ueno, K. Fujii, T. Shinki, Vitamin D and bone, J. Cell. Biochem. 88 (2003) 259–266.
[11] T. Matsumoto, M. Ito, Y. Hayashi, T. Hirota, Y. Tanigawara, T. Sone, M. Fukunaga, M. Shiraki, T. Nakamura, A new active vitamin D3 analog, eldecalcitol, prevents the risk of osteoporotic fractures—a randomized, active comparator, double-blind study, Bone 49 (2011) 605–612.
[12] C.M. Girgis, K.M. Cha, P.J. Houweling, R. Rao, N. Mokbel, M. Lin, R.J. Clifton-Bligh, J.E. Gunton, Vitamin D receptor ablation and vitamin D deficiency result in reduced grip strength, altered muscle fibers, and increased myostatin in mice, Calcif. Tissue Int. 97 (2015) 602–610.
[13] C.M. Girgis, R.J. Clifton-Bligh, M.W. Hamrick, M.F. Holick, J.E. Gunton, The roles of vitamin D in skeletal muscle: form, function, and metabolism, Endocr. Rev. 34 (2013) 33–83.
[14] M. Visser, D.J. Deeg, P. Lips, Longitudinal Aging Study Amsterdam, Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia): the Longitudinal Aging Study Amsterdam, J. Clin. Endocrinol. Metab. 88 (2003) 5766–5772.
[15] M. Bhat, R. Kalam, S.S. Qadri, S. Madabushi, A. Ismail, Vitamin D deficiency-induced muscle wasting occurs through the ubiquitin proteasome pathway and is partially corrected by calcium in male rats, Endocrinology 154 (2013) 4018–4029.
[16] T.J. Wang, M.J. Pencina, S.L. Booth, P.F. Jacques, E. Ingelsson, K. Lanier, E.J. Benjamin, R.B. D'Agostino, M. Wolf, R.S. Vasan, Vitamin D deficiency and risk of cardiovascular disease, Circulation 117 (2008) 503–511.
[17] A.G. Pittas, M. Chung, T. Trikalinos, et al., Systematic review: vitamin D and cardiometabolic outcomes, Ann Intern Med. 152 (2010) 307–314.
[18] T.J. Wang, Q.S. Wells, Vitamin D deficiency and heart failure risk: not so black and white? JACC Heart Fail. 3 (2015) 357–359.
[19] J.M. Calvo-Romero, J.M. Ramiro-Lozano, Metabolic effects of supplementation with vitamin D in type 2 diabetic patients with vitamin D deficiency, Diabetes Metab Syndr. 10 (2016) 72–74.
[20] J.P. McGlade, S. Gorman, G.R. Zosky, A.N. Larcombe, P.D. Sly, J.J. Finlay-Jones, D.J. Turner, P.H. Hart, Suppression of the asthmatic phenotype by ultraviolet B-induced, antigen-specific regulatory cells, Clin. Exp. Allergy 34 (2007) 1267–1276.
[21] I. Karakis, M.P. Pase, A. Beiser, S.L. Booth, P.F. Jacques, G. Rogers, C. DeCarli, R.S. Vasan, T.J. Wang, J.J. Himali, C. Annweiler, S. Seshadri, Association of serum vitamin D with the risk of incident dementia and subclinical indices of brain aging: the Framingham Heart Study, J. Alzheimers Dis. 51 (2016) 451–461.
[22] B. Fattizzo, A. Zaninoni, J.A. Giannotta, F. Binda, A. Cortelezzi, W. Barcellini, Reduced 25-OH vitamin D in patients with autoimmune cytopenias, clinical correlations and literature review, Autoimmun. Rev. 15 (2016) 770–775.
[23] D.E. Lawson, A.A. Paul, A.E. Black, T.J. Cole, A.R. Mandal, M. Davie, Relative contributions of diet and sunlight to vitamin D state in the elderly, Br. Med. J. 2 (1979) 303–305.
[24] E.M. Poskitt, T.J. Cole, D.E. Lawson, Diet, sunlight, and 25-hydroxyvitamin D in healthy children and adults, Br. Med. J. 1 (1979) 221–223.
[25] M.F. Holick, N. Engl. J. Med. 357 (2007) 266–281.
[26] J.A. MacLaughlin, R.R. Anderson, M.F. Holick, Spectral character of sunlight modulates photosynthesis of previtamin D3 and its photoisomers in human skin, Science 216 (1982) 1001–1003.
[27] S. Gorman, N.M. Scott, D.H. Tan, C.E. Weeden, R.C. Tuckey, J.L. Bisley, M.A. Grimbaldeston, P.H. Hart, Acute erythemal ultraviolet radiation causes systemic immunosuppression in the absence of increased 25-hydroxyvitamin D3 levels in male mice, PLoS One 7 (2012), e46006.
[28] L. Mao, Y. Tamura, N. Kawao, K. Okada, M. Yano, K. Okumoto, H. Kaji, Influence of diabetic state and vitamin D deficiency on bone repair in female mice, Bone 61 (2014) 102–108.
[29] J.P. McGlade, S. Gorman, J.C. Lenzo, J.W. Tan, T. Watanabe, J.J. Finlay-Jones, W.R. Thomas, P.H. Hart, Effect of both ultraviolet B irradiation and histamine receptor function on allergic responses to an inhaled antigen, J. Immunol. 178 (2007) 2794–2802.
[30] S. Geldenhuys, P.H. Hart, R. Endersby, P. Jacoby, M. Feelisch, R.B. Weller, V. Matthews, S. Gorman, Ultraviolet radiation suppresses obesity and symptoms of metabolic syndrome independently of vitamin D in mice fed a high-fat diet, Diabetes 63 (2014) 3759–3769.
[31] R.C. Malley, H.K. Muller, M. Norval, G.M. Woods, Dietary vitamin D alters the response of the skin to UVB-irradiation depending on the genetic background of the mice, Photochem Photobiol Sci. 12 (2013) 536–545.
[32] M. Satué, A. Córdoba, J.M. Ramis, M. Monjo, UV-irradiated 7-dehydrocholesterol coating on polystyrene surfaces is converted to active vitamin D by osteoblastic MC3T3-E1 cells, Photochem Photobiol Sci. 12 (2013) 1025–1035.
[33] L.M. Coe, S.A. Tekalur, Y. Shu, M.J. Baumann, L.R. McCabe, Bisphosphonate treatment of type I diabetic mice prevents early bone loss but accentuates suppression of bone formation, J. Cell. Physiol. 230 (2015) 1944–1953.
[34] M.L. Bouxsein, S.K. Boyd, B.A. Christiansen, R.E. Guldberg, K.J. Jepsen, R. Muller, Guidelines for assessment of bone microstructure in rodents using micro-computed tomography, J. Bone Miner. Res. 25 (2010) 1468–1486.
[35] K.D. Healy, J.B. Zella, J.M. Prahl, H.F. DeLuca, Regulation of the murine renal vitamin D receptor by 1,25-dihydroxyvitamin D3 and calcium, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 9733–9737.
[36] M.K. Watson, A.W. Stern, A.L. Labelle, S. Joslyn, T.M. Fan, K. Leister, M. Kohles, K. Marshall, M.A. Mitchell, Evaluating the clinical and physiological effects of long term ultraviolet B radiation on guinea pigs (*Cavia porcellus*), PLoS One 17 (2014), e114413.
[37] E. Sallander, U. Wester, E. Bengtsson, Edström D. Wiegleb, Vitamin D levels after UVB radiation: effects by UVA additions in a randomized controlled trial, Photodermatol Photoimmunol Photomed. 29 (2013) 323–329.
[38] R. Bouillon, J. Eisman, M. Garabedian, M. Holick, J. Kleinschmidt, T. Suda, I. Terenetskaya, A. Webb, Action Spectrum for Production of Previtamin D3 in Human Skin. CIE Technical Report 174, Commission Internationale de l'Eclairage (CIE) Central Bureau, Vienna, Austria, 2006.
[39] M. Norval, L.O. Björn, F.R. de Gruijl, Is the action spectrum for the UV-induced production of previtamin D3 in human skin correct? Photochem Photobiol Sci. 9 (2010) 11–17.
[40] E. Havinga, Vitamin D, example and challenge, Experientia 29 (1973) 1181–1193.
[41] H.J.C. Jacobs, J.W.J. Gielen, E. Havinga, Effects of wavelength and conformation on the photochemistry of vitamin D and related conjugated trienes, Tetrahedron Lett. 22 (1981) 4013–4016.
[42] W.G. Dauben, B. Disanayaka, J.H. Funhoff, B. Zhou, B.E. Kohler, D.E. Schilke, Polyene 2^1A_g and 1^1B_u states and the photochemistry of previtamin D_3, J. Am. Chem. Soc. 113 (1991) 8367–8374.
[43] S. Gliesing, M. Reinbacher, H.D. Ilge, D. Faßler, Wellenlangeneffekte auf die quantenausbuten ten photoisomerisierung von pravitamin D_3, Z. Chem. 29 (1989) 21–22.
[44] E. Thieden, H.L. Jørgensen, N.R. Jørgensen, P.A. Philipsen, H.C. Wulf, Sunbed radiation provokes cutaneous vitamin D synthesis in humans—a randomized controlled trial, Photochem. Photobiol. 84 (2008) 1487–1492.
[45] R. McKenzie, R. Scragg, B. Liley, P. Johnston, J. Wishart, A. Stewart, R. Prematunga, Serum 25-Hydroxyvitamin-D responses to multiple UV exposures from solaria: inferences for exposure to sunlight, Photochem Photobiol Sci. 11 (2012) 1174–1185.
[46] M.C. Cheung, P.B. Spalding, J.C. Gutierrez, W. Balkan, N. Namias, L.G. Koniaris, T.A. Zimmers, Body surface area prediction in normal, hypermuscular, and obese mice, J. Surg. Res. 153 (2009) 326–331.
[47] G.A. Knaysi, G.F. Crikelair, B. Cosman, The rule of nines: its history and accuracy, Plast. Reconstr. Surg. 41 (1968) 560–563.
[48] R.S. Weinstein, J.L. Underwood, M.S. Hutson, H.F. DeLuca, Bone histomorphometry in vitamin D-deficient rats infused with calcium and phosphorus, Am. J. Phys. 246 (1984) 499–505.
[49] L. Lieben, R. Masuyama, S. Torrekens, R. Van Looveren, J. Schrooten, P. Baatsen, M.H. Lafage-Proust, T. Dresselaers, J.Q. Feng, L.F. Bonewald, M.B. Meyer, J.W. Pike, R. Bouillon, G. Carmeliet, Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D-induced inhibition of bone mineralization, J. Clin. Invest. 122 (2012) 1803–1815.
[50] G. Carmeliet, V. Dermauw, R. Bouillon, Vitamin D signaling in calcium and bone homeostasis: a delicate balance, Best Pract Res Clin Endocrinol Metab. 29 (2015) 621–631.
[51] A.M. Lee, R.K. Sawyer, A.J. Moore, H.A. Morris, P.D. O'Loughlin, P.H. Anderson, Adequate dietary vitamin D and calcium are both required to reduce bone turnover and increased bone mineral volume, J. Steroid Biochem. Mol. Biol. 144 (2014) 159–162.