

## The Benefits of the Combination of Vitamin D3, K2 Supplements, and UV-B Exposure for Increasing Bone Density: A Simple Solution for Bone Health

Sindy Cornelia Nelwan<sup>1\*</sup>, Udijanto Tedjosasongko<sup>1</sup>, Satiti Kuntari<sup>1</sup>, Daniel Haryono Utomo<sup>2</sup>, Tania Saskianti<sup>1</sup>, Mega Moeharyono Puteri<sup>1</sup>, Devi Dharmawan<sup>1</sup>, Yufita Fitriani<sup>1</sup>, Pradita Agung Kurnia<sup>1</sup>, Amalia Ramadhani Mufida<sup>1</sup>, Nadhia Zahria Fajrin<sup>1</sup>, Retno Pudji Rahayu<sup>3</sup>, Nunthawan Nowwarote<sup>1,4</sup>

1. Department of Pediatric Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya-Indonesia.

2. Department of Dental Forensic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya-Indonesia.

3. Department of Oral Pathology and Maxillofacial, Faculty of Dental Medicine, Universitas Airlangga, Surabaya-Indonesia.

4. Centre de Recherche des Cordeliers, INSERM UMRS 1138, Molecular Oral Pathophysiology, Université de Paris, Sorbonne Université, Paris-France.

### Abstract

Bones and teeth require nutrients to maintain optimal bone density. Environmental and habitual factors affect 20-40% of bone density in addition to several nutrients such as vitamin D, especially for UV-B exposures which are essential for bone growth.

Research on the impact of nutrition on bone strength has historically geared towards minerals, vitamin D, protein, and vitamin K. This study aimed to examine the role of vitamin D3, K2 supplements, and UV-B exposure on bone density, reflected by osteoblast cell formation. Pre- and post-experimental laboratory studies were conducted to Wistar rats (*Rattus norvegicus*), which were divided into six groups. As much as 0.00045 mg/day of vitamin D and 0.81 mcg/day of K2 were administered. Besides, 290-315 nm of UV-B was administered 3 times a week for 25 minutes each. With the HE (hematoxylin eosin) test, the level of osteoblast cell was calculated after 21 days of treatment.

Descriptive test results showed that the highest mean score was obtained from the vitamin D3+K2 group. The ANOVA test result showed sig < 0.05 (p < 0.05), which indicated a significant difference. **This research shows that** the combination of vitamin D3 and K2 significantly increased osteoblast cell formation.

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### Introduction

One of the most common metabolic conditions worldwide, both in developed and developing countries, is vitamin D deficiency.<sup>1</sup> Vitamin D deficiency is a global problem and impacts developed and developing countries, in addition to subtropical and temperate regions. Vitamin D deficiency in Asians of all ages may be disregarded. Vitamin D deficiency seldom occurs in areas with abundant sunshine.<sup>2</sup> It is an essential nutrient for optimal health and bone

growth.<sup>3</sup> Vitamin D deficiency has been found to be prominent in several countries at rates of 5.9% (US), 7.4% (Canada), and 13% (Europe). Many countries have reported very high incidences of low vitamin D. The South East Asian Nutrition Survey (SEANUTS) found that, in Indonesia, vitamin D deficiency was discovered in 49.3% of children aged 2.0-12.9 years (analysis data).<sup>4,5</sup>

Vitamin D is primarily supplied endogenously, and it has been found that 90% needed for humans comes from exposure to the sun.<sup>6</sup> UV-B light (290-315 nm) irradiation of the skin from direct exposure to sunlight is the main vitamin D source for humans.<sup>1</sup> Vitamin D is among the molecules responsible for bone metabolism. It plays an important part in bone synthesis, where it is responsible for increasing calcium's absorption rate as the primary mineral in bones.<sup>3,6</sup>

#### \*Corresponding author:

Sindy Cornelia Nelwan,  
Department of Pediatric Dentistry, Faculty of Dental Medicine,  
Universitas Airlangga, Surabaya-Indonesia.  
E-mail: [sindy-c-n@fkg.unair.ac.id](mailto:sindy-c-n@fkg.unair.ac.id)

The World Health Organization (WHO) acknowledged vitamin D deficiency as a public health problem in March 2020 and attributed it to the coronavirus disease 2019 (COVID-19). The virus transmitted from animals to humans. The novel coronavirus spread out in late 2019, and it was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as a part of the subfamily  $\beta$ -coronaviruses. It shares 79.5% of SARS-CoV's genetic sequence. The disease resulting from SARS-CoV-2 is coronavirus disease 2019 (COVID-19).<sup>7</sup> This virus has swiftly spread across the globe and caused the outbreak of a highly infectious respiratory disease. Various countries then began to implement COVID-19 protocols in accordance with WHO recommendations such as handwashing, avoiding of crowd or in-person meeting, social distancing, and limitation in activities outside of the house. Isolation procedures set by the government include self-isolation for individuals, communities, and even entire cities. Countries have implemented large-scale social restrictions and even lockdowns. As a result, many offices, both government and private, have implemented "work from home" for their employees.<sup>8</sup> These lifestyle changes have caused a rise in indoor activities and instead decrease sunlight exposure among children and adolescents.<sup>9</sup> The outbreak of COVID-19 has also caused notable changes in child's day to day life. Most in-person class meetings have been substituted with home-schooling and online learning activities. In other words, all schools instruct students to 'stay at home'; as a result, children might have reduced sunlight exposure.<sup>10,11</sup>

Beside vitamin D, previous studies have found that supplementation of vitamin K may be advantageous for the metabolism and quality maintenance of bone, particularly when it comes to improving density. Vitamin K is needed for the regulation of calcium intake in the human body. Vitamin K assists the integration of calcium into bone. There are three proteins that depend on vitamin K in bone: protein S, osteocalcin [bone gamma-carboxyglutamic acid (GLA) protein], and matrix GLA protein.<sup>12</sup>

Combining vitamin D and K will have desired effects on calcium homeostasis that improves bone quality. Vitamin D increases the calcium absorption rate. Vitamin K regulates calcium which promotes calcification of bone and

activate osteocalcin to promote the buildup of calcium in bones and teeth. Vitamin K2 also transports calcium into muscle to construct bones and keeps it in the right place.<sup>3</sup>

Previous studies suggest that vitamin D increases concentrations of bone protein dependent on vitamin K and triggers in vitro bone formation with the stimulation of osteoblast-specific gene expression (osteocalcin) controlled by 1.25(OH)D at a transcriptional level through the responsive element of 1.25(OH)D within the promoter of the osteocalcin gene. The effect of vitamin K of bone mineralization varies due to plasma 25(OH)D concentration<sup>13</sup>. Observational studies are in line with the hypothesis that bone mineralization may be supported by ideal vitamin D and K concentrations.<sup>13</sup>

Bone is a mineralized connective tissue made up of four different types of cells: osteocytes, bone lining cells, osteoblasts, and osteoclasts. Osteoblasts play an essential role in bone formation, turnover, and tissue repair. Osteoblasts have three functional stages in vivo and in vitro: bone matrix synthesis/maturation, mineralization, proliferation.<sup>14,15</sup>

Bone formation requires some minerals, ions, and vitamins D and K for the formation of collagen and crystals, metabolism of bone and cartilage, and/or homeostasis of phosphate and Ca. The abnormalities of bone happen because of micro damage associated with lack of nutrients. With that said, nutrients are necessary for bone and teeth to grow and develop.<sup>16</sup>

## Materials and methods

This was an in vivo experimental laboratory study using osteoblast cells on 48 white rats (*Rattus norvegicus*). The inclusion criteria were 50-200 grams of male Wistar rats (2-3 months old) which were generally in good condition and adapted for seven days. As much as 1000 IU of Vitamin D3 (Doctor's Best, US) and 45 mcg of Vitamin K2 (Doctor's Best, US) supplements were administered to the rats once a day. Besides the vitamins, the rats were also given 290-315 nm UV-B exposure (Phillips) for 25 minutes, three times a week. These 48 white rats (*Rattus norvegicus*) were split into six group for the experiment:

- Group I : Control group
- Group II : UV-B exposure
- Group III : Vitamin D3 and K2 supplements

- Group IV : UV-B exposure, vitamins D3 and K2 supplements
- Group V : K2 supplements
- Group VI : UV-B exposure + K2 supplements

**Procedures**

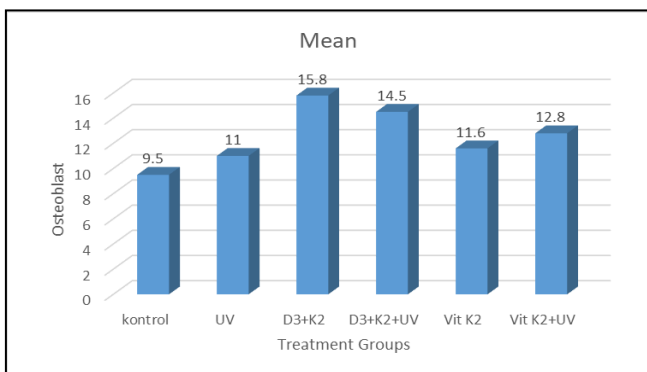
The technique used was extraction of the rats' incisors to find osteoblast cells from bone healing remedies after tooth extraction. Test animals that were in the adaptation process were given food, water, and libitum with husks being replaced every other day. They were given intraperitoneal anesthesia using ketamine combined with 0.1 mL/10 g body weight (BW) xylazine. After the anesthesia was administered, the tooth extraction began. The incisor areas were sterilized with iodine and then the left lower incisors were extracted using forceps.<sup>17</sup> Then, intervention was given for 21 days. The results were collected from alveolar bone healing areas post-extraction. The osteoblasts cells were obtained by performing an HPA examination under the microscope.

**Research Ethics**

This study has obtained research ethical approval from the Commission of Ethical Clearance, Faculty of Dental Medicine, Universitas Airlangga with the Ethics Code of 371/HRECC.FOOM/VIII/2020.

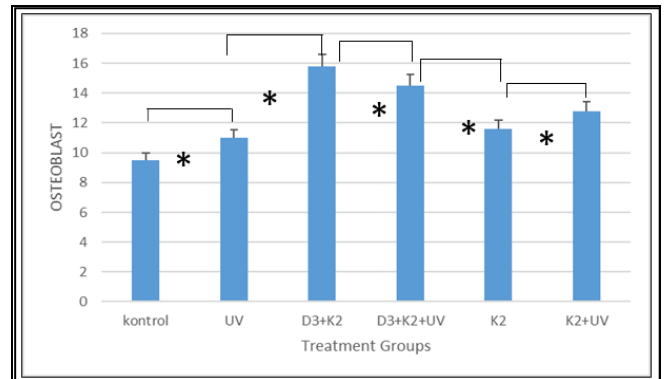
**Results**

The samples showed the total amount of osteoblast cell deposition. The samples comprised of six groups with a total of six rats in each group. The highest osteoblast level was found in Group III (Vit D3+Vit K2), followed by the D3+K2+UV group, the Vit K2+UV group, the Vit K2 group, the UV group, and finally the control group (Group I) (see Figure 1).



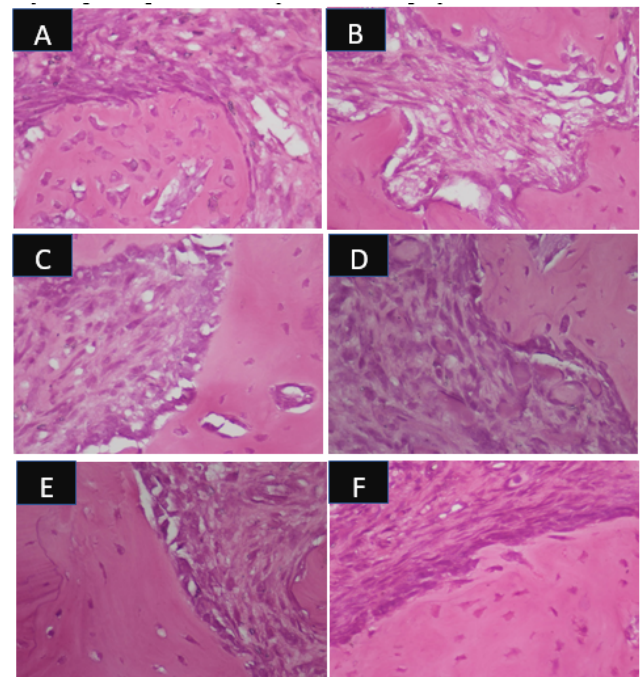
**Figure 1.** Mean of the osteoblast cell deposition in each group.

The statistical analysis with the SPSS 24.0 showed that the ANOVA test resulted significant differences for post-treatments groups ( $p \leq 0.05$ ) with 95% Confidence Interval (see Figure 2).



**Figure 2.** Osteoblast cell deposition in each group.

Histopathological images of osteoblast deposition from each group can be seen below.



**Figure 3.** (A) Control, (B) UV-B, (C) Vitamin D3 + K2, (D) Vitamin D3 + K2 + UV-B, (E) Vitamin K2, (F) Vitamin K2 + UV-B.

**Discussion**

Bone is a type of mineralized connective tissue that contains four types of cells: osteoclasts, bone-lining cells, osteoblasts, and

osteocytes. Osteoblasts come from mesenchymal stromal cell (MSC) progenitors and present themselves during the process of active bone formation. Osteoblasts have an integral place in bone physiology due to their role in the formation of bone, turnover, tissue repair, controlling bone remodeling, and regulation of phosphate homeostasis. The maturation or differentiation of osteoblastic cells occurs in a sequential succession beginning with osteoprogenitors followed by pre-osteoblasts, osteoblasts, and finally osteocytes. Osteoblasts go through three functional stages in vivo and in vitro: proliferation, bone matrix synthesis or maturation, and mineralization. The membrane expression of certain function-related proteins, known as markers, has been studied on precursor cells during differentiation.<sup>14,15,18</sup>

Bone quality can be measured based on mineral composition, size, shape, collagen content, and remodeling process. Osteoblasts and osteoclasts play a role in bone apposition and resorption, respectively. This constant reconstruction of tissue is known as remodeling. The cells involved in the remodeling process include progenitor cells, osteoblast, osteocytes, and osteoclast. Bone mineral content and quality determine bone strength. Bone strength weakens with abnormalities in bone construction, such as bone matrix protein deterioration and bone micro-architecture, high or low bone-turnover rate, and buildup of micro-damage. These conditions can worsen even more due to insufficient nutrients. Nutrients are necessary for the bone and teeth to grow and develop. Bone formation requires several minerals for bone-forming (Mg, Zn, P, Ca), ions (e.g. citrate, Cu, carbonate, and Mn), and vitamins (e.g. vitamins C, D, K) in charge of the formation of crystal and collagen, the metabolism of cartilage and bone, and/or homeostasis of Ca and phosphate.<sup>19-22</sup>

Vitamin D has exponentially increased as a result of a worldwide prevalence of vitamin D deficiency (VDD) in this pandemic.<sup>23</sup> Vitamin D has three sources: UVB radiation-dependent endogenous production, dietary supplements, and nutritional source. This study observed the osteoblast levels and obtained optimal intakes of vitamin D and K2 doses and UVB irradiation.

Vitamin D has many effects on osteoblast cells. Active metabolite vitamin D (1,25(OH)2D3) can affect human osteoblast growth and differentiation; stimulate bone formation,

mineralization, and osteogenic differentiation from human MSC to the osteoblast lineage; inhibit adipocyte formation by way of Wnt pathway stimulation; control remodeling through induction of NF- $\kappa$ B ligand's receptor activator; regulate phosphate homeostasis by increasing FGF23; affect energy metabolism through osteocalcin (BGLAP) stimulation; and improve the bone's response to mechanical loads through f mitogen-activated protein kinase signaling stimulation. It can also encourage the formation of osteoclast in co-cultures of osteoblastic and hematopoietic cells in mice. Osteoblastic cells express RANKL and OPG, thereby controlling osteoclast generation. After activation, vitamin D binds to the vitamin D receptor (VDR), a representative receptor for the steroid receptor family, which mediates the homeostasis of calcium and phosphate. It has been demonstrated that vitamin D can act directly on the growth and materialization of bone, as well as in bone remodeling.<sup>18,24-26</sup>

Vitamin K behaves like a cofactor in the carboxylation of glutamic acid (Glu) to Gla. It is also the metabolically active form of osteocalcin, which is able to join and release calcium in the extracellular matrix. Vitamin K2 also directly impacts bone cells, encouraging osteoblast genesis and inhibiting osteoclast differentiation. Vitamin K2 could bond to the steroid and xenobiotic receptor (SXR), thus causing increased expression of multiple bone matrix elements. Low vitamin K intake and high undercarboxylated OC (unOC) levels are linked to a higher possibility for bone fragility. Previous studies by Sato et al. and Ambrożewicz et al. showed MK-7 to have the highest bioavailability and the most notable impact on OC carboxylation in human and enhanced osteoblast proliferation.<sup>27-29</sup>

The administration of the combination of vitamin D and K is thought to have favorable effects on the homeostasis of calcium, improve bone quality, and lower the risk of fractures. Vitamin D encourages production of proteins dependent on vitamin K, while vitamin K triggers bone metabolism proteins. This study reviewed the impact of vitamin D3, K2 supplements, and UVB radiation on osteoblast differentiation. The vitamin D3 and K2 supplement combination produced the highest amount of osteoblast compared to just vitamin D3 or K2 on their own. This is in line with a study by Ballegoijen<sup>13</sup> that

showed the combination of vitamin D and K2 induced important biochemical changes in bone. Another study showed that vitamin D increases concentrations of vitamin K-dependent bone protein, bone cell parameters in murine osteoblast cell lines and human primary osteoblasts, and triggers the formation of bone *in vitro* with osteoblast-specific gene expression stimulation.<sup>27-30</sup>

Ultraviolet irradiation is the primary vitamin D synthesis source in the skin. UVB irradiation increases vitamin D levels and stimulates osteoblast genes. This study showed the combination of D3+K2 and UVB irradiation had lower osteoblast levels than the combination of D3 + K2. However, this study found the UVB radiation wavelength around 280-320 nm. Morita et al. reported the highest production of vitamin D was at a UVB radiation wavelength of 282 nm, while not at 320nm.<sup>6</sup> Another study by Lancaster et al. reported that the addition of K2 +1.25D to osteoblasts led to an increase in bone mineralization, however it did not consistently shift all the osteoblast maturation parameters related to bone formation.<sup>15</sup> Further studies should evaluate the wavelength of UVB radiation.<sup>30</sup>

## Conclusions

The combination of vitamin D3, K2 supplements, and UV-B (290-315 nm) increases osteoblast as a bone formation marker.

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## Declaration of Interest

The authors report no conflict of interest.

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