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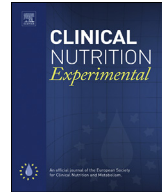

Sunlight exposure vs. vitamin D supplementation on bone homeostasis of vitamin D deficient rats

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SUMMARY

Background & aim of the work: Vitamin D deficiency is highly prevalent in our locality and worldwide. Two major sources of Vit D replenishment are available; sun exposure and dietary supplementation. This study compared sun exposure with vitamin D3 supplementation on restoring bone homeostasis in a vitamin D deficient (VDD) versus vitamin D sufficient (VDS) rat models.
Methods: Vitamin D deficient-normocalcemic diet was used for 6 weeks to produce a group of VDD rats (n = 21), then were subdividedinto three equal subgroups: direct sunlight exposure subgroup (Sd subgroup), vitamin D3 treated subgroup (Tt subgroup), and a Control subgroup (Ct group). Normal rats (group II) (n = 14) were fed on normal vitamin D-diet for equal period and then subdivided into two equal subgroups: direct sunlight exposure subgroup (Ss subgroup), and a control (C) subgroup. For all of the subgroups, Exposures and supplementations were continued for 10 days, then the plasma 25 hydroxy vitamin D3, parathyroid hormone, calcium, phosphorus levels and alkaline phosphatase activity were estimated and femur bones were used to prepare histopathological sections.
Results: The sun-exposed vitamin D deficient group showed a significant reduction of parathyroid hormone more than that in vitamin D supplemented group vs. VDD controls (67.69 ± 13.18 and 78.93 ± 8.31 vs. 86.05 ± 9.67 pg/ml, *respectively*), while sun-exposed vitamin D sufficient group showed an insignificant change (15.56 ± 2.73 vs. 16.84 ± 3.16 pg/ml). Furthermore, the improvement of osteoid area and the reduction of trabecular

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separation in the bone sections among vitamin D deficient rats, after sunlight exposure, were better.

Conclusions: Sunlight-exposure have a more positive effect on bone structure and homeostasis than vitamin D supplementation and control. This effect was more with vitamin D deficient than vitamin D sufficient rats.

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1. Introduction

Vitamin D deficiency is a common metabolic condition worldwide [1]. Twenty five hydroxy vitamin D (25 OH Vit D3) level more than 20 ng/mL (50 nmol/L) is considered sufficient, between 15 and 20 ng/mL (37.5–50 nmol/L) is insufficient, while its level below 15 ng/mL (37.5 nmol/L) is deficient [2]. Despite the sunny weather in the Middle East, spanning latitudes from 12 N to 42 N that allow for vitamin D synthesis year round, this region reports individuals with some of the lowest levels of vitamin D and the highest rates of hypovitaminosis D worldwide [3]. In Egypt there are inaccurate prevalence rates. In the United Arab Emirates (UAE) a study reported 50% of pregnant women being deficient, while in Saudi Arabia, a study found 59% of healthy 4–15-year-old school children to be deficient and 28% insufficient in vitamin D [4].

Solar UV-B (wavelengths of 290–315 nm) irradiation of the skin via direct sunlight exposure is the primary source of vitamin D for most people [1,5]. Dietary sources of vitamin D are limited. They include oily fish such as salmon, mackerel, sardines and some fish oils such as cod liver oil, and egg yolks. In addition, some foods including milk and some cereals, orange juice, some yogurts, and margarine are fortified in many countries, by variable amount and forms of vitamin D [6].

Despite vitamin D fortification and dietary counseling efforts that emphasize ingestion of vitamin D-containing dietary sources, vitamin D deficiency occurs in a pandemic fashion. This suggested the value and roles of sun exposure as a preventive and curative remedy for vitamin D deficiency. On other hand, Overexposure to both natural and artificial UV radiations encounters some adverse effects such as photo-aging, photo-allergic, and phototoxic reactions as well as carcinogenesis, including life-threatening melanomas [7].

The present study was carried out to demonstrate and compare the effects of sun light exposure versus vitamin D supplementation on bone structure and plasma levels of some bone-related homeostatic hormones and minerals on vitamin D deficient and sufficient rats.

2. Material and methods

2.1. Animals

A total of 35 healthy male albino rats were used. Shortly after weaning, 3 to 4 week old rats (body weight 78.70 ± 10.2 g) were used in accordance with an experimental protocol approved by the ethics committee of College of Applied Medical Sciences, King Saud University (reference no: CAMS 52-35/36). All of the rats were bred in normal dark/light cycle and 25 °C room temperature in animal house. The rats were divided primarily into two groups: group I (n = 21) were fed a customized; vitamin D deficient, normal calcium and phosphorus diet (Custom AIN-93G, Bio-Serve, USA) (Table 1) [8] for 6 weeks in covered cages to limit direct exposure to florescent light. Group II (n = 14) rats were fed on a normal balanced growth diet (AIN-93G, Bio-Serve, USA) [9] in a normally lighted room.

After 6 weeks, rats in group I were subdivided into 3 equal subgroups. The direct sunlight-exposed subgroup (**Sd subgroup**). The vitamin D_3 treated subgroup (**Tt subgroup**) that was treated with oral vitamin D_3 (MUP co, Egypt), with canola oil as 3 ml of a 27 ug/ml vitamin D_3 solution administered

Table 1
General futures of composition of rat's diets.

Item	Group I diet	Group II diet
Protein	18.1%	18.1%
Fat	7.1%	7.1%
Carbohydrate	59.3%	59.3%
Total energy	3.74 kcal/gm	3.74 kcal/gm
Fiber	4.8%	4.8%
Ash	2.2%	2.2%
Calcium	5.1 g/kg	5.1 g/kg
Phosphorus	2.8 gm/kg	2.8 gm/kg
Vitamin D3	0–50 IU/kg	1000 IU/kg

together with an equal amount of pure canola oil divided into 3 doses over the treatment period [10]. The control subgroup (**Ct subgroup)** that was treated with 3 ml of canola oil without vitamin D for 10 days. One rat from Ct group died during the study. In another set of experiments, the rats in group II were subdivided into two equal subgroups; the direct sun-light exposed subgroup (**Ss subgroup)** that was exposed to sunlight and fed the same diet of group II (vitamin D 1000 IU/kg) for 10 days and the control (**C) subgroup,** which received no intervention but continued feeding on the group II diet.

2.2. Sun light exposure

The Sd and Ss subgroups were exposed to direct sunrays from 1:30 to 2 pm during May 2015 in Riyadh Saudi Arabia (latitude 24°N). Body hair of the sunlight-exposed rats was not shaved. No forms of sun-screen or protection were used to protect the sun-exposed groups; rather, they were left in an open field in separate cages to reduce thermal exhaustion and sweating [11].

2.3. Blood sampling

Blood samples (0.5 ml/rat) were obtained from tail veins before the treatment period. At the end of the study, after overnight fasting, blood (5–6 ml/rat) was collected via cardiac puncture from the rats while under deep anesthesia (3–5% isoflurane in a vaporizer chamber), followed immediately by cervical dislocation as an appropriate and humane method of euthanasia. Blood samples were collected in green toped, heparinized tubes (Greiner Bio-One Germany) and then centrifuged for 15 min at approximately 500 rpm. The separated plasma supernatant was stored at −20 °C until the time of measurement. Repeated freezing and thawing were avoided.

2.4. Biochemical analysis

1 **Plasma 25 hydroxy vitamin D and parathyroid hormone (PTH) levels**: ELISA kits (Mybiosource USA) were used according to the manufacturer's protocols [12,13].
2 **Calcium (Ca) levels and alkaline phosphatase enzyme activity**: A calcium colorimetric assay kit (Randox UK) and was used according to the manufacturer's protocol [13].
3 **Phosphate (P) level:** Via a colorimetric assay kit (Biovision USA) [13].
4 **Alkaline phosphatase enzyme (ALP) activity**: An alkaline-phosphatase colorimetric assay kit (Randox UK) was used according to the manufacturer's protocol. The enzyme activity was assessed at 3 time points, and the average was used as the final result [14].

2.5. Bone specimen and histology

After euthanasia of the rats, the right femur bone was used for the studies of bone structure. The bone specimens were fixed for two days in 0.5% cyanuric chloride in methanol containing 1% (0.1 M) N-methylmorpholine [15], decalcified in 10% formic acid formalin and were routinely processed for hematoxylin and eosin (HE) staining. The slides were then examined under a compound light

microscope and histopathological changes were assessed. The regions of interest (ROIs) were randomly selected within three sections per limb and viewed under the microscope at 400× magnification [11]. Digital images of histological sections were taken and analyzed using image J software, an image processing program designed for scientific multidimensional images [16], (Image J, National Institutes of Health, USA). Image J tools were used to measure osteoid area and areas of bone marrow spaces (trabecular separation) in the selected ROIs. Two ROIs from each sample were selected and analyzed.

2.6. Statistical analysis

The data were presented as the mean ± SD. Statistical significance before and after was determined by paired Student's "t" test. An ANOVA with a post hoc test was used to analyze the differences in multiple comparisons. P values < 0.05 were considered to be significant. Regarding the statistical analysis of the bone histopathological findings, Wilcoxon t test was used to test the statistical significance of before–after changes in osteoid area and trabecular separation. For the statistical analyses, SPSS version 22 for Windows (SPSS Inc. Chicago, IL, USA) was used.

3. Results

3.1. Bone health in growing rats fed a vitamin D deficient diet

Figure 1 shows the histopathological differences between rats fed on a customized vitamin D deficient diet (group I) vs. the rats of vitamin D sufficient group (group II). Grossly cut sections revealed no significant histopathological changes. Microscopic examinations revealed moderate osteoporotic changes with reduced thickening of the bone cortex associated with widely separated bone trabeculae containing bone marrow element in group I rats, while the bone structures in the control group revealed benign bone tissue and trabeculae (Fig. 1A_{b-c}). Tools of image J software were used to objectively measure osteoid area and trabecular separation in both groups and revealed a significant reduction of the osteoid area and a significant increase in trabecular separation in ROIs selected from

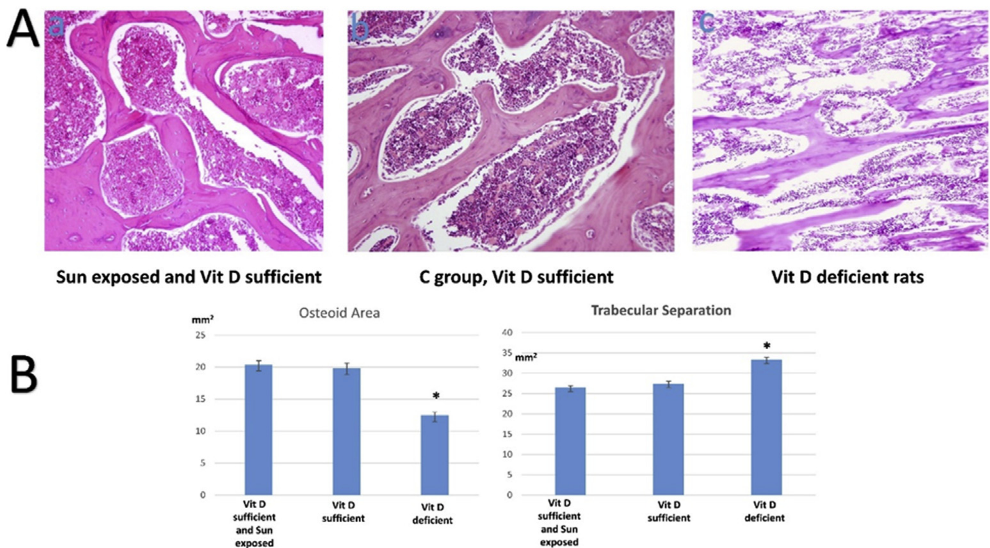

Fig. 1. Histopathological bone changes among sun-exposed, vitamin D sufficient subgroup (Aa), vitamin D sufficient control (Ab), and vitamin D deficient subgroup (Ac), in addition to histomorphometric deference in osteoid area and trabecular separation among them (B). $^{*}P \leq 0.05$.

group I (*Group II vs. Group I*: *19.82* ± *0.82 vs. 12.48* ± *0.46* mm^2 *and 27.46* ± *0.59 vs. 33.40* ± *0.52* mm^2, *respectively*, $P < 0.05$) (Fig. 1B).

3.2. Changes in plasma levels of 25 hydroxyvitamin D3, parathyroid hormone, calcium, phosphate and alkaline phosphatase among all subgroups

Levels of 25 Hydroxyvitamin D3 in plasma of all subgroups before and after 10 days period of treatment were shown in Table 2. No significant changes were detected in the levels of 25 hydroxyvitamin D3 during the treatment period in all groups.

Additionally all measured parameters in all subgroups after treatment period were reported in Table 3. There is a significant reduction of parathyroid hormone level in vitamin D deficient-sun exposed group (Sd group) vs. vitamin D deficient control (Ct group), (67.69 ± 13.18 vs. 86.05 ± 9.67 pg/ml, $P < 0.05$) and versus vitamin D supplemented rats (67.69 ± 13.18 vs. 78.93 ± 8.31 pg/ml, $P < 0.05$).

3.3. Effects of sunlight exposure on vitamin D deficient and vitamin D sufficient rats

Figure 2A shows structural changes of the femur of vitamin D deficient rats after 10 days sun exposure. Trabecular thickness and number were improved together with a reduction of bone marrow spaces. Objective measurements of osteoid area and trabecular separation in Sd subgroups before and after sun exposure revealed a significant increase in osteoid area and a significant decrease in trabecular separation (Fig. 2B) (*Sd Before vs. Sd After*: *13.47* ± *0.35 vs. 20.94* ± *0.17* mm^2 *and 30.73* ± *0.80 vs. 21.98* ± *0.67* mm^2, *respectively*, $P < 0.05$).

As seen above in Fig. 1$A_{a,b}$ there is an insignificant difference in the histological structure and objective histomorphometric parameters between the vitamin D sufficient (C subgroup) and the vitamin D sufficient-rats with sun exposure (Ss subgroup) (Fig. 1B).

3.4. Effects of vitamin D supplementation on vitamin D deficient rats

In regards to the effect of a given dose of vitamin D on bone structure, microscopic examination revealed some improvement in the moderate osteoporotic picture of before-sections, while the histomorphometric measures of after-ROIs revealed significant increase in osteoid areas (*Tt Before vs. Tt After*: *14.75* ± *0.49 vs. 19.78* ± *0.5*, $P < 0.05$) and an insignificant change in trabecular separation (Fig. 3).

4. Discussion

Vitamin D deficiency is a major metabolic syndrome that mainly worsens the bone health in the form of bone growth retardation and the development of classic signs and symptoms of rickets, osteopenia and osteoporosis [17]. Rat models of hypovitaminosis D are frequently used in vitamin D research. Dietary deprivation of vitamin D and keeping animals away from sunlight are used as traditional models [12]. The variable roles of sunlight exposure on bone structure and health are best studied in animal models as they preclude any human related behavioral and cultural factors that may affect vitamin D and calcium metabolism. In this study, a rat model of normocalcemic hypovitaminosis D was utilized by feeding weanling rats on a customized vitamin D deficient diet. All vitamin D

Table 2
Levels of 25 hydroxyvitamin D3 in plasma of all subgroups before and after 10 days of treatment.

Group	Mean ± SD		*P* (Paired T test)
	Before (ng/ml)	After (ng/ml)	
Sd group	14.40 ± 3.19	14.19 ± 2.72	0.680
Tt group	14.33 ± 3.43	14.05 ± 3.08	0.356
Ct group	18.26 ± 6.38	15.59 ± 2.45	0.421
Ss group	35.16 ± 10.54	30.06 ± 13.14	0.300
C group	31.68 ± 10.40	36.84 ± 9.16	0.352

Table 3
Study variables among study subgroups after treatment period. One-way ANOVA with post hoc test was used to test the significant difference. $^*P < 0.05$.

Groups	25 OH Vit D3 (ng/ml)	PTH (pg/ml)	Calcium (mg/dl)	Phosphorus (mg/dl)	ALP (U/l)
Sd group	14.19 ± 2.72	67.69 ± 13.18	6.48 ± 2.12	1.42 ± 0.42	156.14 ± 43.31
Tt group	14.05 ± 3.08	78.93 ± 8.31	6.68 ± 1.92	1.17 ± 0.62	171.00 ± 17.61
Ct group	15.59 ± 2.45	86.05 ± 9.67	5.32 ± 1.28	1.33 ± 0.32	182.62 ± 61.83
Ss group	30.06 ± 13.14	15.56 ± 2.73	9.92 ± 0.48	3.64 ± 0.83	75.96 ± 35.42
C group	36.84 ± 9.16	16.84 ± 3.16	10.24 ± 0.92	3.67 ± 1.13	58.50 ± 11.47
F (between groups)	13.25	19.72	14.05	20.23	12.81
P value	0.000*	0.000*	0.000*	0.000*	0.000*
Tukey HSD					
Sd vs. Ct	1.000	0.011*	1.000	0.996	0.996
Tt vs. Ct	1.000	0.850	0.532	1.000	0.999
Sd vs. Tt	1.000	0.029*	0.703	1.000	0.907
Ss vs. C	0.651	0.111	0.999	1.000	0.990

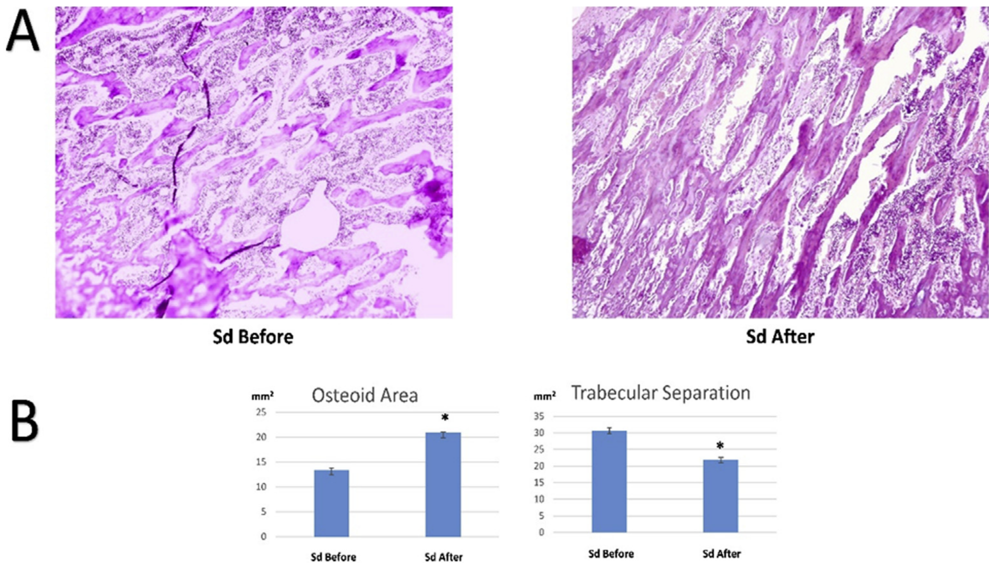

Fig. 2. Histopathological changes in vitamin D deficient rats of Sd subgroup before and after 10 day period of sun exposure (A) and significant improvement in osteoid area and trabecular separation before and after exposure (B). $^*P \leq 0.05$.

deficient subgroups (Sd, Tt & Ct) showed variable degrees of secondary hyperparathyroidism (PTH > 65 pg/ml cutoff value, [18]), normocalccemia (Calcium within the normal range 5.3–13 mg/dl [19]), hypophosphatemia and increased alkaline phosphatase enzyme activity. In addition, histopathological and histomorphometric measures revealed moderate osteoporotic changes with a significant reduction of bone area and a wide separation of bone trabeculae. These results were partially in line with those of Kollenkirchen et al. [20], the only difference being normal phosphate and PTH in normocalcemic vitamin D deficient rats in the Kollenkirchen model. The main cause of this difference was dietary lactose, which increases passive intestinal calcium absorption [21]. Furthermore, these metabolic and histological changes in normocalcemic vitamin D deficient growing rats were similar to those reported by Lester et al. [22].

The absence of measured of 1, 25 dihydroxyvitamin D levels is a considerable limitation to this study even though current endocrine societies guidelines recommend an assessment of the father metabolite for screening of vitamin D deficiency. Serum levels of 1,25-dihyroxyvitamin D have little or no relation

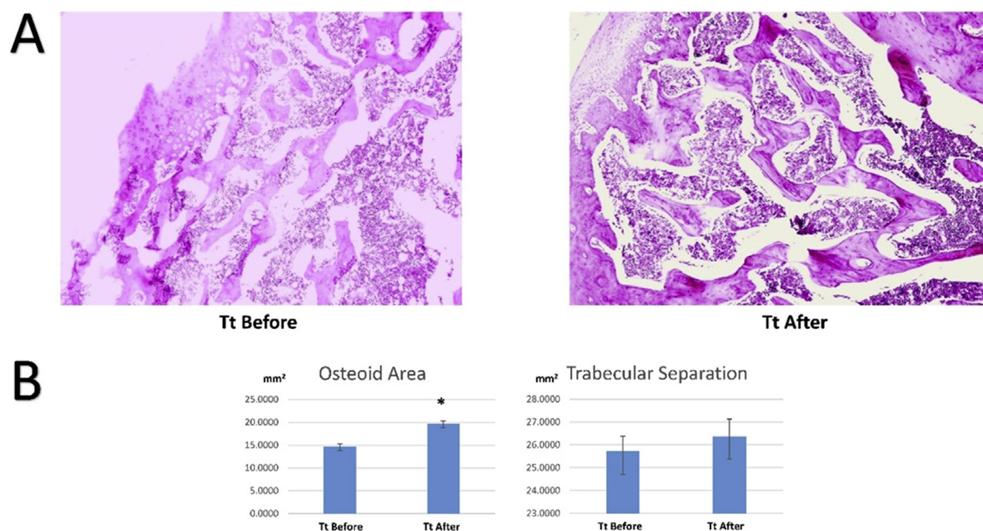

Fig. 3. Histopathological (A) and histomorphometric parameters (B) changes in vitamin D deficient rats of Tt subgroup before and after treatment period. *$^{*}P \leq 0.05$.*

to vitamin D stores but rather are regulated primarily by parathyroid hormone levels, which in turn are regulated by calcium and/or vitamin D [23]. The post-interventional insignificant decrease in the level of 25 hydroxyvitamin D in both sun exposed and vitamin D_3 supplemented subgroups may be due to the relatively short treatment period or the consumption of the vitamin D pool in restoring bone structure. The latter explanation is supported by significant positive changes in histological and histomorphometric parameters measured before and after the treatment period. These findings regarding sun exposure were in line with previous studies conducted in Nigeria [15,24]. These changes were more prominent with the sun-exposed rather than vitamin D supplemented subgroup. However Watson et al. [25] reported that exposing guinea pigs to artificial UVB radiation for 6 months significantly increased their circulating serum 25-hydroxyvitamin D3 levels, and that this increase was sustainable over time.

Hyperparathyroidism was a common finding in all of the subgroups fed a vitamin D deficient diet. Active vitamin D metabolites decrease PTH synthesis in vitro and in vivo [26,27]. It was concluded that 1,25-dihydroxyvitamin D, independent of changes in intestinal calcium absorption and serum calcium, can represses the transcription of PTH by binding to the vitamin D receptor, which heterodimerizes with retinoic acid X receptors to bind vitamin D-response elements within the PTH gene. In addition, 1,25-dihydroxyvitamin D regulates the expression of calcium-sensing receptors to indirectly alter PTH secretion. As a result, reduced concentrations of calcium-sensing and vitamin D receptors and altered mRNA-binding protein activities within parathyroid cells, increase PTH secretion in addition to the more widely recognized changes in serum calcium, phosphorus, and 1α,25-dihydroxyvitamin D [28]. These facts may in part explain of hyperparathyroidism here in this study i.e., a result of low vitamin D metabolites in the plasma of group I rats. Despite normal Ca levels, hypovitaminosis D caused a state of hyperparathyroidism, evidencing the relatively narrow range of regulation of PTH secretion by extracellular calcium [29]. The significant effect of sunlight on the level of PTH in the Sd subgroup vs. the Ct subgroup (67.69 ± 13.18 vs. 86.05 ± 9.67 pg/ml, $P < \pm 0.05$), and the insignificant change in the Ss subgroup vs. C subgroup (15.56 ± 2.73 vs. 16.84 ± 3.16 pg/ml) can be explained in the light of above data by the UVB-induced increase in vitamin D metabolites and calcium sensing. UVB photons with energies of 290–315 nm are absorbed by 7-dehydrocholesterol in the skin and converted to previtamin D_3. Previtamin D_3 undergoes a rapid transformation into vitamin D_3 within the plasma membrane. These changes in vitamin D-molecules could alter membrane permeability and possibly open up a pore to permit the entrance and exit of ions including calcium, ensuring the availability of vitamin D and Ca,

leading to a reduction of PTH secretion [30,31]. However, excessive exposure to sunlight does not result in vitamin D intoxication, as both previtamin D_3 and vitamin D_3 are photolyzed to several noncalcemic photoproducts [32]. This is not the case with pharmacological vitamin D supplementation.

The mean changes in pre- and post-sun exposure 25 hydroxyvitamin D levels were 0.29 ng/mL for the Sd subgroup and 5.10 ng/mL for the Ss subgroup, but they were not statistically significant. These data were consistent with human results reported by Lee et al. [33], despite the exposure of their study subjects to sunlight for 20 min for four weekends (8 days). This large difference in response of vitamin D deficient rats to sunlight vs. vitamin D sufficient rats is also appear in PTH levels (i.e., significant change of PTH between the Sd and the Ct subgroups, while, insignificant change of PTH in the Ss vs. the C subgroups). Furthermore, the histological changes were more pronounced in the vitamin D deficient subgroups (Figs. 1 and 2). This demonstrates that vitamin D deficient bones respond in a different manner to sunrays in a mechanism that may extend beyond vitamin D- and calcium-related actions. In line with this hypothesis, we observed a significant reduction of PTH levels together with an insignificant reduction of alkaline phosphatase activity between the Sd and Tt subgroups.

In conclusion, the effects of sun-exposure on bone health may extend beyond normalization of 25 hydroxy vitamin D plasma levels to a more positive effect on bone structure and the hormones that control bone mass, which may be mediated by other mechanisms. Future studies should do more clarification about this issue.

Conflict of interest statement

The author declares that he has no competing interests.

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