RESEARCH ARTICLE

Effects of sunlight exposure vs. commercial vitamin D supplementation on the bone health of vitamin D deficient rats

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ABSTRACT

This study compared sun exposure with commercial vitamin D3 supplementation in restoring bone health in a vitamin D deficient rat model. 35 healthy weaned male albino rats were divided into two groups. Vitamin D deficient rats (n=21) were subdivided into: an Sd group (n=7) that was directly exposed to sun-light, a Tt group (n=7) that was treated with commercial vitamin D3 with oil, and a Ct group (n=7) that was treated with canola oil without vitamin D. Normal diet fed rats were subdivided into: a Cs group (n=7) that was exposed to sunlight and fed a normal vitamin D chow and a control (C) group(n=7). Plasma 25 hydroxy vitamin D3, parathyroid hormone, calcium, phosphorus levels and the enzymatic activity of alkaline phosphatase were estimated and femur bones were used to prepare histopathological sections. The sun-exposed groups showed a significant reduction in levels of parathyroid hormone (67.69±13.18 vs 86.05±9.67 pg/mL, $P < 0.05$) in addition to an improvement of osteoid area and a reduction of trabecular separation in the bone sections. In a conclusion, sun-exposure have a more positive effect on bone structure and hormones that control bone mass rather than normalization of vitamin D.

Key words: Sunlight exposure, vitamin D supplementation, vitamin D deficiency, bone histology.

INTRODUCTION

Vitamin D deficiency is a highly prevalent metabolic condition worldwide (Holick and Chen 2008).Despite the sunny weather in the Middle East, spanning latitudes from 12N to 42N 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 (Fuleihan, 2009). For instance, in Saudi Arabia the prevalence of vitamin

D deficiency was estimated to be 29% of studied population, which also showed a 22.7% relative insufficiency state (Alsuwadia *et al.,* 2013). This major metabolic problem affects populations at all developmental stages, including pregnant women, neonates, infants, children and adolescents, adults, and the elderly. In addition, maternal vitamin D levels strongly correlate with neonatal levels (El Rifai *et al.,* 2014). Vitamin D deficiency in children causes growth retardation, which classically manifest with symptoms of rickets (Huldschinsky, 1919). In adults, vitamin D deficiency will precipitate and exacerbate osteopenia and osteoporosis and increase the risk of pathological bone fractures (Larsen *et al.,* 2004; Bakhtiyarova *et al.,* 2006). Vitamin D deficiency not only causes metabolic bone disease among children and adults but also may increase the risk of many common chronic diseases such as diabetes, obesity, autoimmune diseases, cancers and cardiovascular disease (Holick, 2004).

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 (Holick, 2002; Holick and Chen, 2008). Dietary sources of vitamin D are limited. They include oily fish such as salmon, mackerel, and sardines; 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 (Holick *et al.,* 1992; Tangpricha *et al.,* 2003).

Despite vitamin D fortification and dietary counseling efforts that emphasize ingestion of vitamin D from 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. The wearing of traditional clothes, the deliberate avoidance of the sun and an indoor life-style may in part explain the high prevalence of vitamin D deficiency (Alsuwadia *et al.,* 2013; Green *et al.,* 2015). Additionally, vitamin D resistance resulting from calcium deficiencies has also been described in some populations in year-round sunny areas, which can lead to hypovitaminosis D (Green *et al.,* 2015).

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 (Lester *et al.,* 1982; Stavenuiter *et al.,* 2015). The variable roles of sunlight exposure and dietary vitamin D supplementation 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. Taking these factors into consideration, this study was carried out to demonstrate and compare the effects of sun light exposure vs vitamin D_3 supplementation on bone histology and plasma levels of some bone-related hormones and minerals, such as 25 hydroxy vitamin D_3, Parathyroid hormone, calcium, phosphorus and alkaline phosphatase in a rat model of hypovitaminosis D.

MATERIALS AND METHODS

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 usedin accordance with an experimental protocol approved by the ethics committee of College of Applied Medical Sciences, King Saud University. All of the rats were bredin light-temperature-controlled animal housing (12-h light, 12-h dark cycle, temperature approximatly 25 C^o, respectively). The rats were divided primarily into two groups: those in group I (n=14) were fed on a normal balanced growth diet (AIN-93G, Bio-Serve, USA) (Reeves *et al.,* 1993) in a normally lit room. The remaining rats in group II (n=21) were fed a customized; vitamin D deficient, normal calcium and phosphorus diet (Custom AIN-93G, Bio-Serve, USA)(table 1)(Bio-Serv 2015) for 6 weeks in covered cages to limit direct exposure to florescent light.

After 6 weeks, the rats in group I were subdivided into two subgroups; the Ss group was exposed to sunlight and fed same diet of group I (vitamin D 1000 IU/kg) for 10 days and the C group, which received no intervention but continued feeding on the group I diet. In another set of experiments, rats in group II were subdivided into 3 subgroups. The Sd group (n=7) was directly exposed to sun-light. The Tt group (n=7) was treated with commercial vitamin D_3 with canola oil as 3 ml of a 27 ug/ml vitamin D_3 solution (Fleet *et al.,* 2008)(MUP co, Egypt) administered together with an equal amount of pure canola oil divided into 3 doses over the treatment period. The Ct group (n=7) was treated with 3 ml of canola oil without vitamin D for 10 days. One rat from Ct group died during the study.

Table 1: General futures of composition of rat's diets (16)

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 gm/kg	5.1 gm/kg
Phosphorus	2.8 gm/kg	2.8 gm/kg
Vitamin D3	1000 IU/kg	0-50 IU/kg

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). 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.

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 minutes at approximately 500 rpm**.** The separated plasma supernatant was stored at -80°C untill the time of measurement. Repeated freezing and thawing were avoided**.**

Biochemical analysis:

1. Measuring plasma 25 hydroxy vitamin D and parathyroid hormone (PTH) levels: ELISA kits (Mybiosource USA) were used according to the manufacturer's protocols (Shuai *et al.,* 2008; Stavenuiter *et al.,* 2015).

2. Measuring calcium (Ca) levels and alkaline phosphatase enzyme activity: A calcium colorimetric assay kit (Randox UK) and was used according to the manufacturer's protocol (Stavenuiter *et al.,* 2015).

3. Measurement of phosphate (P) level: Via a colorimetric assay kit (Biovision USA)(Stavenuiter *et al.,* 2015).

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 (Randox 2015).

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 (Yoshiki, 1973), 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 (Iyanda and Iheakanwa, 2014), and digital images of histological sections were taken and analyzed using image J software (Egan *et al.,* 2012) (Image J, National Institutes of Health, USA). Image J tools described by Egan *et al.,* (2) 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.

Statistical Analysis:

The data were presented as the mean ± SD. Statistical significance before and after was determined by paired Student's "t" test. An independent samples T test was used to compare any groups of interest while 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. For the statistical analyses, SPSS version 22 for Windows (SPSS Inc. Chicago, IL, USA) was used**.**

RESULTS

Bone health in growing rats fed a vitamin D deficient diet:

Fig. 1. shows the histopathological differences between rats fed on a customized vitamin D deficient diet (group II) vs. the rats of the control group (group I). Grosslycut 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 II rats, while the bone structures in the control group revealed benign bone tissue and trabeculae (figure $1A_{b-c}$). 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 group II *(Group I vs. Group II: 19.82±0.82 vs. 12.48±0.46mm²* and *27.46±0.59 vs. 33.40±0.52 mm²,* respectively, *P < 0.05)(fig. 1B).*

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. Additionally all measured parameters in all subgroups after treatment period were reported in table 3.

Effects of sunlight exposure on vitamin D deficient and vitamin D sufficient rats:

Fig. 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 mm²* and *30.73±0.80 vs. 21.98±0.67 mm²,* respectively, *P < 0.05).*

Fig.$1A_{a,b}$ demonstrates an insignificant difference in the histological structure and objective histomorphometric parameters between the vitamin D sufficient subgroup and the vitamin D sufficient-group with sun exposure (Ss subgroup) (figure 1B).

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).

Table 2: levels of 25 Hydroxyvitamin D3 in plasma of all subgroups before and after 10 daysperiod of treatment.

Group	Mean ± SD		*P (Paired T test)*
	BEFORE (ng/ml)	AFTER (ug/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 groups after treatment period presented as means ±SD. One-way ANOVA test with post hoc test was calculated. ** 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	86.05±9.67	5.32±1.28	1.33±0.32	182.62±61.83
Ct group		15.59±2.45	78.93±8.31	6.68±1.92	1.17±0.62	171.00±17.61
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	36.84±9.16	10.24±0.92	3.67±1.13	58.50±11.47
F (between groups)		13.25	59.72	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	0.451	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	0.011*	0.999	1.000	0.990

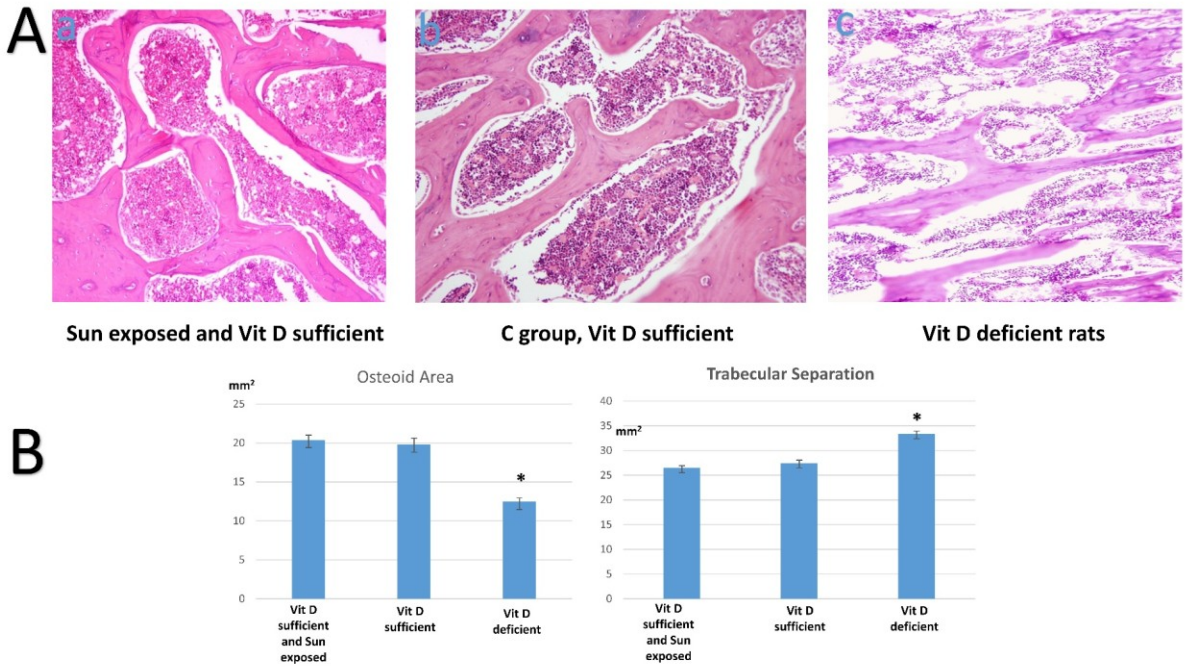

Fig 1: Histopathological bone changes among sun-exposed, vitamin D sufficient subgroup (Aa), vitamin D sufficient control (Ab), and vitamin D deficient subgroup (Ac), in addition tohistomorphometric deference in osteoid area and trabecular separation among them (B). ** p≤0.05.*

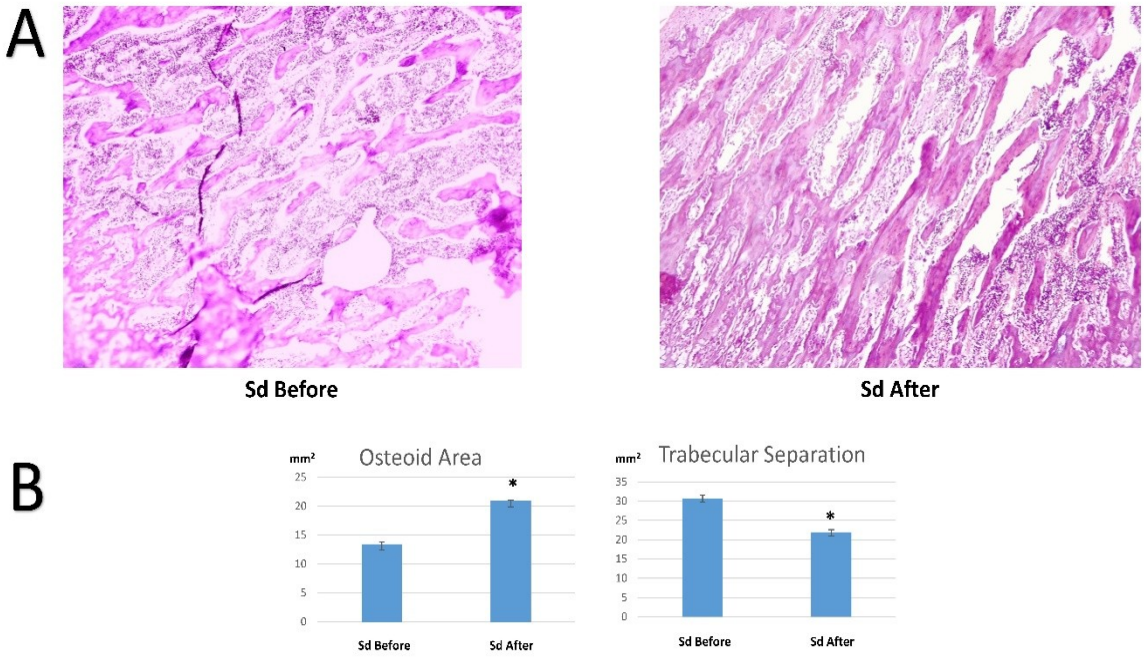

Fig. 2: Histopathological changes in vitamin D deficient rats of Sd subgroup before and after 10 day period of sun exposure (A) and significant improvement in osteoid area and trabecular separation before and after exposure (B). ** p≤0.05*

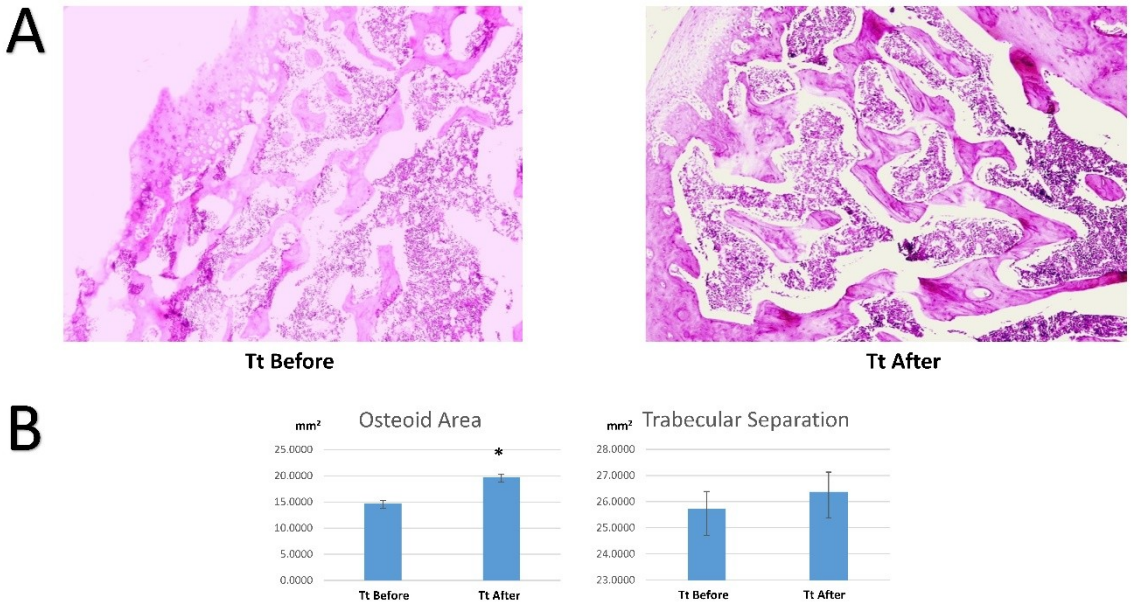

Fig. 3: Histopathological (A) and histomorphometric parameters (B) changes in vitamin D deficient rats of Tt subgroup before and after treatment period. ** p≤0.05*

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 (Huldschinsky, 1919; Larsen *et al.,* 2004). 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 deficient subgroups (Sd, Tt & Ct) showed variable degrees of secondary hyperparathyroidism (PTH > 65 pg/ml cutoff value, (Aloia *et al.,* 2006)), normocalccemia (Calcium within the normal range 5.3-13 mg/dl (Johnson-Delaney 1996), 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.,* (1991), 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 intestinalcalcium absorption (Bronner, 1987). Furthermore, these metabolic and histological changes in normocalcemic vitamin D deficient growing rats were similar to those reported by Lester *et al.,* (1982).

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 to vitamin D stores but rather are regulated primarily by parathyroid hormone levels, which in turn are regulated by calcium and/or vitamin D (Holick *et al.,*, 2011). 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 (Iyanda and Iheakanwa 2014, Iyanda, 2014). These changes were more prominent with the sun-exposed rather than the commercial vitamin D supplemented subgroup.

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 (Silver 1985, Silver *et al.,* 1986). 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-dihydroxy-vitamin D (Kumar and Thompson, 2011). 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 II rats. Despite normal Ca levels, hypovitaminosis D caused a state of hyperpara-thyroidism, evidencing the relatively narrow range of regulation of PTH secretion by extracellular calcium (Mundy and Guise, 1999). The limited effect of sun light on the level of PTH in the Sd subgroup vs. the Tt subgroup (67.69±13.18 vs. 86.05±9.67, $P < 0.05$) and in Ss subgroup vs. C subgroup (15.56±2.73 vs.36.84±9.16, $P < 0.05$) 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 (Tian *et al.,* 1993; Wacker and Holick, 2013). 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 (Holick *et al.,* 2007). This is not the case with pharmacological vitamin D supplementation.

The mean changes in pre- and post-sun exposure 25 hydroxyvitamin D levels were-0.21 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.,* (2012), 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., insignificant change of PTH between the Sd and the Ct subgroups, while, significant reduction of PTH in the Ss vs. the C subgroups). However, the histological changes were more pronounced in the vitamin D deficient subgroup (figure 2, 3). This demonstrates that vitamin D deficient bones respond in a different manner to sunrays in a manner 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.

CONCLUSIONS

In a-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.

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