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ORIGINAL ARTICLE

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Evaluation of vitamin D status and its correlation with gonadal function in children at mini-puberty

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Summary

Context: The effects of Vitamin D on reproductive function in adults have gained interest. Studies have demonstrated some associations. Hypothalamic-pituitary-gonadal axis is activated during the first 6 months of life, called as mini-puberty. This HPG activation is important for future gonadal function. There are no data regarding the association of gonadal hormones and 25(OH)D levels at mini-puberty. Demonstration of any association would form the basis for studies that will search for the effects of 25(OH)D on gonadal hormones at mini-puberty.

Objective: To characterize the associations between 25(OH)D levels and gonadal hormones at mini-puberty.

Design: Cross-sectional cohort analysis.

Patient(s) or other participant(s): A total of 180 (94 boys and 86 girls) healthy appropriate-for-gestational-age neonates were included.

Main outcome measure(s): 25(OH)D, LH, FSH, total testosterone, oestradiol, AMH and inhibin B levels were measured at postnatal 30-45 days. All infants were divided into three groups including vitamin D deficiency (<10 ng/mL), vitamin D insufficiency (10-20 ng/mL) and vitamin D sufficiency (>20 ng/mL). Correlations between vitamin D status and reproductive hormones were analysed.

Result(s): Total testosterone level was higher (mean: 0.52 ± 0.32 vs 0.26 ± 0.2 ng/mL; *P*: 0.008) and inhibin B was lower in 25(OH)D deficient than sufficient girls (mean: 21.2 ± 15.71 vs 53.25 ± 47.25 pg/mL; *P*: 0.021).

Conclusion(s): A modest effect of 25(OH)D was identified on total testosterone and inhibin B in girls at mini-puberty. The 25(OH)D may have an effect on gonadal function during early life. Randomized controlled trials could clarify the importance of vitamin D on gonadal hormones at mini-puberty.

KEYWORDS

AMH, inhibin B, mini-puberty, reproduction, sex steroids, vitamin D

1 | INTRODUCTION

Vitamin D deficiency has been linked to various health disorders.[1] While the role of vitamin D deficiency in reduced bone mass is evident, its relation to other health disorders is the subject of an extended controversy. In addition to its classic effects on bone homoeostasis, many studies have supported that vitamin D receptors (VDRs) are present on various non-musculoskeletal organs and tissues.[2] Most recent evidence from human and animal studies also suggests that vitamin D has a potential role in the physiology of reproductive function in both genders.[3,4] This interaction between vitamin D and reproduction is attributed to the presence of both

VDR and vitamin D metabolizing enzymes (CYP2R1, CYP27B1 and CYP24A1) in the reproductive organs.[5,6]

Although several studies have investigated the effects of vitamin D on reproductive function and gonadal hormone production in men and women, little is known about the mechanism by which vitamin D affects reproductive physiology.[7] Studies showed that male VDR knockout mice have significant gonadal insufficiency, decreased sperm count and motility, and histological abnormalities of testes.[6,8] Similarly, uterine hypoplasia, impaired folliculogenesis, reduced aromatase gene expression and gonadal insufficiency were found in most of the female VDR null mutant mice.[9,10] Many human studies have also demonstrated an association between serum vitamin D concentrations and gonadal hormone production.[11-14] But some other studies have failed to document this correlation.[15-18] Therefore, the literature is conflicting and complete characterization of the association of vitamin D levels with the gonadal function remains to be elucidated.

Mini-puberty, which is described as a transient activation of the hypothalamo-pituitary-gonadal (HPG) axis within the first few months of life, is associated with a temporary activation of gonadal hormone production in both boys and girls.[19] Gonadotropin levels are low in both sexes at birth. They start to increase after the postnatal age 7 days and make a peak at around 1-3 months of postnatal life. This is a critical time period in which gonadal hormones rise up to the pubertal levels and measurements of those hormone levels at this period will give important clues with respect to future gonadal function. HPG activity then gradually decreases and remains quiescent during childhood until reactivation at puberty.[19] LH and FSH decrease to prepubertal levels at 6-9 months in boys. In girls, LH levels decrease at the same time as in boys while FSH remains elevated up to the age of 3-4 years.[19] To our knowledge, there is not any study that investigated the effect of vitamin D on gonadal function at mini-puberty period. This is the first study that investigated the role of vitamin D in male and female gonadal function at mini-puberty period with particular emphasis on production of sex steroids and gonadal peptide hormones.

2 | SUBJECTS AND METHODS

2.1 | Study participants and design

This study was performed from June 1, 2017 to December 31, 2017. We analysed data from a prospective cohort of 180 (94 boys and 86 girls) unselected, healthy infants aged 30 to 45 days. All infants were thoroughly examined twice, at 3 days after birth and again at 30-45 days of life. As recommended, vitamin D supplements were given at a dose of 400 IU/d to all participants, beginning a few days after birth, in accordance with the American Academy of Pediatrics (AAP). Blood samples were taken at 30-45 days of age. All infants were full-term and appropriate-for-gestational-age. A detailed medical assessment was performed and medical history was obtained from parents of all subjects. Birth weight and length of the participants were also recorded.

Infants who were born prematurely, large for gestational age (LGA), small for gestational age (SGA), have medical problems such as thyroid hormone dysfunction, genetic syndromes and other endocrine disorders, and those who used medications that might potentially influence the biomedical assessments and alter vitamin D metabolism were excluded from the study.

2.2 | Anthropometric measurements

Length was measured with a portable infantometer (Seca infantometer; Germany) to the nearest 0.1 cm. Weight was measured on a digital scale (Seca baby scale; Germany) to the nearest 0.005 kg. The same team of trained doctors performed all examinations, and all methods of measuring were standardized at workshops attended by all examiners. The mean of three measurements was calculated for all measures.

2.3 | Ethical aspects

The study was performed according to the Helsinki II declaration and approved by the local Ethical Committee of Medical Faculty at the Medipol University (Approval number: 326). Only one attempt was made to obtain a blood sample from each infant. Written informed consent was obtained from the parents of all enrolled children.

2.4 | Laboratory methods

Nonfasting peripheral venous blood samples were taken from an antecubital vein between 08.00 and 10.00 AM. Samples were separated by centrifugation and stored protected from light at −80°C until analysis.

2.5 | Assays

Electrochemiluminescence immunoassay (ECLIA) on the Elecsys autoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland) was used to quantify serum 25-hydroxyvitamin D (25(OH)D) concentration. Interassay coefficients of variation (CV) were 5.2% and intra-assay CV was 6.8%. The detection limit was 3 ng/mL. Competitive ECLIA on the cobas® 6000 analyzer (Roche Diagnostics) was used to quantify serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), total testosterone (TT) and oestradiol (E2). The lowest limits of detection were 0.1 mIU/mL for LH, 0.1 mIU/mL for FSH, 0.025 ng/mL for TT and 5 pg/mL for E2. The intra-assay CV was 7.3%, and the inter-assay CV was 5.2% for LH, 6.1%, and 8.1% for FSH, 7.1%, and 5.3% for TT, 6.3%, and 5.4% for E2, respectively. Serum anti-müllerian hormone (AMH) was analysed using the ECLIA on the cobas e 411 analyzer (Roche Diagnostics). The detection limit was 0.01 ng/mL. Intra- and interassay CV were less than 1.43% and less than 0.51%. Serum inhibin B was analysed using enzyme-linked immunosorbent assay (ELISA) (Ansh Labs, TX, USA). The detection limit was 1.6 pg/mL. Intra- and interassay CV were less than 4.4% and less than 3.8%.

TABLE 1 Characteristics of the all infants according to 25(OH)D status

25(OH)D	<10 ng/mL n: 29		10-20 ng/mL n: 59		>20 ng/mL n: 92		*P*[a]
Age (d)	38.24 ± 4.63		39.42 ± 4.05		39.35 ± 3.29		0.228
Gender							
Male	15	51.7%	37	62.7%	42	45.7%	0.123[b]
Female	14	48.3%	22	37.3%	50	54.3%	
Birth weight (g)	3327.07 ± 350.47		3285.54 ± 389.65		3260.73 ± 445.47		0.750
Weight (g)	4251.79 ± 442.02		4311.09 ± 574.97		4259.2 ± 531.07		0.826
Height (cm)	52.36 ± 2.34		52.77 ± 2.45		52.94 ± 2.33		0.525

25(OH)D, 25-hydroxyvitamin D.
Data are presented as mean ± SDS. Values are significant at $P < 0.05$.
[a]ANOVA test.
[b]Chi square test.

2.6 | Statistical analysis

Subjects were divided into three groups: vitamin D sufficiency (vitamin D > 20 ng/mL), vitamin D insufficiency (vitamin D 10-20 ng/mL) and vitamin D deficiency (vitamin D < 10 ng/mL). If a measured hormone concentration was below the limit of detection for the given assay, it was expressed as the limit of detection. Statistical analyses were repeated after excluding undetectable values, but this did not change our results. In addition to descriptive statistics (mean, standard deviation), one-way analysis of variance (ANOVA) test was used for group comparisons of normally distributed variables. Kruskal-Wallis test was used for intergroup comparisons of non-normally distributed variables, Dunn's multiple comparison test was utilized in the comparison of subgroups, and Chi square test was performed for the evaluation of qualitative data. A *P*-value of <0.05 was considered statistically significant. Statistical analysis was performed using the program NCSS 2007 (Number Cruncher Statistical System, Kaysville, UT, USA).

3 | RESULTS

3.1 | Participants' characteristics

A total of 180 infants (94 boys, 86 girls) participated in this study. All the infants were divided into three groups including vitamin D deficiency (<10 ng/mL), vitamin D insufficiency (10-20 ng/mL) and vitamin D sufficiency (>20 ng/mL). No statistically significant difference was found with respect to age, birth weight, current weight and height between groups ($P > 0.05$). The clinical characteristics of the subjects according to vitamin D status are shown in Table 1. Their average age was 39.75 ± 3.79 days, and the mean serum 25(OH)D concentration was 21.48 ± 12.1 ng/mL. Out of 180 infants, 29 (16.1%) had vitamin D deficiency, 59 (32.8%) had vitamin D insufficiency, 92 (51.1%) had a sufficient level.

3.2 | LH, FSH and gonadal hormone levels of the boys and girls at 30-45 days

LH, FSH and gonadal hormones levels in boys and girls at the age of 30-45 days are presented in Table 2 and Figure 1. The distribution of LH and FSH levels with respect to postnatal ages in boys and girls are also shown in Figure 2.

3.3 | Associations of 25(OH)D with LH, FSH and gonadal hormones in boys

A total of 94 boys were included in the study. All the boys were divided into three groups including vitamin D deficiency (<10 ng/mL), vitamin D insufficiency (10-20 ng/mL) and vitamin D sufficiency (>20 ng/mL). No statistically significant difference was found with respect to age, birth weight, current weight and height between groups ($P > 0.05$). The average age of boys was 39.46 ± 4.1 days, and the mean serum 25(OH)D concentration was 20.92 ± 13.02 ng/mL. About 15.9% had vitamin D deficiency, 39.6% had vitamin D insufficiency and 44.6% had sufficient level. The subgroups were compared in terms of the LH, FSH and gonadal hormones. No significant correlation was found between 25(OH)D levels and gonadal hormones in three groups. The correlation between vitamin D and LH, FSH and gonadal hormones in groups and hormone profile of boys with respect to 25(OH)D levels is shown in Table 3 and Figure 3.

TABLE 2 LH, FSH and gonadal hormones levels in boys and girls at age of 30-45 days

	Boys n: 94	Girls n: 86	*P*
LH (IU/L)	6.21 ± 2.85	1.29 ± 1.56	0.001
FSH (IU/L)	2.50 ± 1.37	6.73 ± 4.90	0.001
E2 (pg/mL)	8.26 ± 8.03	9.46 ± 8.78	0.472
TT (ng/mL)	1.45 ± 0.85	0.33 ± 0.26	0.001
AMH (ng/mL)	60.42 ± 31.83	1.67 ± 2.25	0.001
Inhibin B (pg/mL)	200.79 ± 64.14	56.19 ± 61.11	0.001

25(OH)D, 25-hydroxyvitamin D; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, oestradiol; TT, total testosterone; AMH, anti-müllerian hormone.

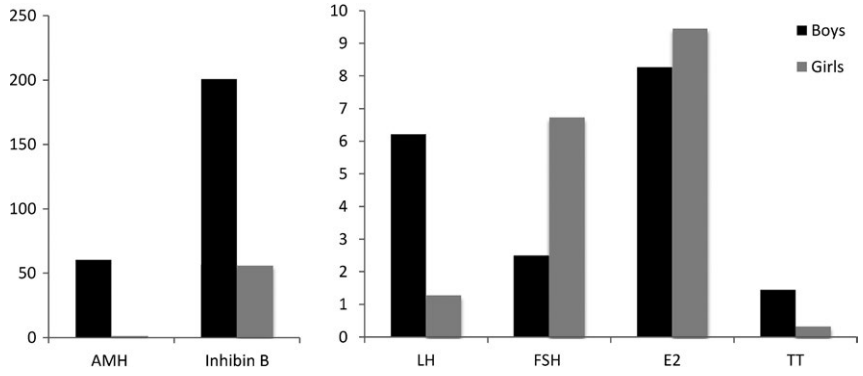

FIGURE 1 Mean FSH, LH and gonadal hormone levels in boys and girls

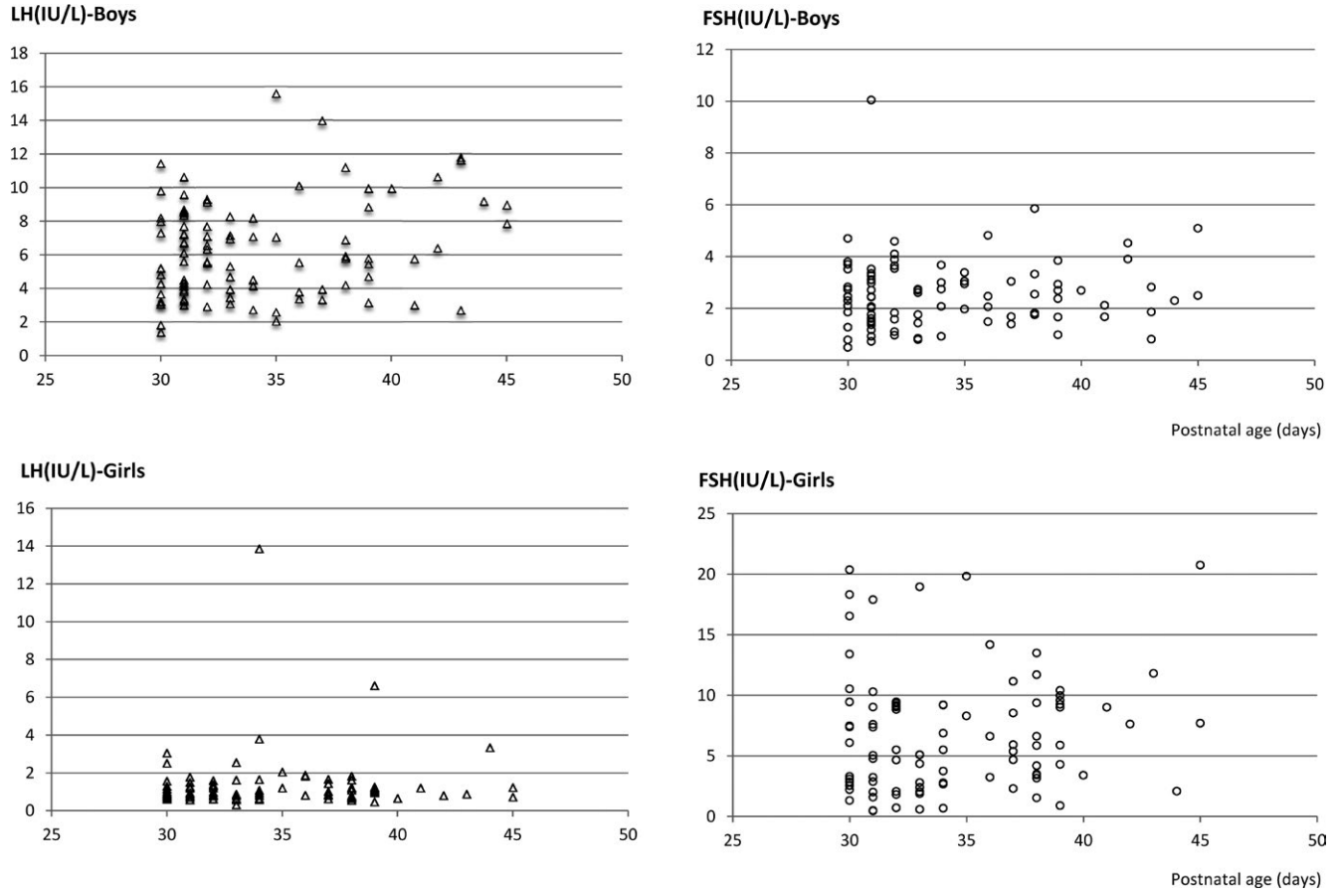

FIGURE 2 Distribution of LH and FSH levels with respect to postnatal ages in boys and girls

3.4 | Associations of 25(OH)D with LH, FSH and gonadal hormones in girls

A total of 86 girls were included in the study. All the girls were divided into three groups including vitamin D deficiency (<10 ng/mL), vitamin D insufficiency (10-20 ng/mL), and vitamin D sufficiency (>20 ng/mL) as described above. No statistically significant difference was found with respect to age, birth weight, current weight and height between groups ($P > 0.05$). Their average age was 40.05 ± 3.42 days, and the mean serum 25(OH)D concentration was 22.09 ± 11.05 ng/mL. About 16.2% had vitamin D deficiency, 25.5% had vitamin D insufficiency and 58.1% had sufficient level. The subgroups were compared in terms of the LH, FSH and gonadal hormones. No significant correlation was found between 25(OH)D levels and LH, FSH, E2 and AMH in three groups. But there was a statistically significant difference between TT levels of the three groups ($P = 0.007$). >20 ng/mL vitamin D group showed a significantly low TT levels compared to <10 ng/mL and 10-20 ng/mL vitamin D groups ($P = 0.003$, $P = 0.025$- Dunn's multiple comparison test). No statistically significant difference was noticed between TT levels of <10 and 10-20 ng/mL vitamin D groups ($P = 0.122$). Moreover, there was a statistically significant difference between

TABLE 3 Association of 25(OH)D with LH, FSH and gonadal hormones in boys and girls

	<10 ng/mL		10-20 ng/mL		>20 ng/mL	
25(OH)D	Boys (n: 15)	Girls (n: 14)	Boys (n: 37)	Girls (n: 22)	Boys (n: 42)	Girls (n: 50)
LH (IU/L)	5.92 ± 2.93	1.39 ± 0.71	6.32 ± 3.03	0.94 ± 0.38	6.29 ± 2.77	1.41 ± 2.03
FSH (IU/L)	2.43 ± 1.25	8.08 ± 6.01	2.63 ± 1.77	4.73 ± 2.58	2.35 ± 0.98	7.32 ± 5.27
E2 (pg/mL)	9.68 ± 8.69	8 ± 5.98	9.07 ± 8.48	9.27 ± 7.02	7.32 ± 7.72	9.69 ± 9.6
TT (ng/mL)	1.37 ± 0.37	0.52 ± 0.32	1.51 ± 1.14	0.36 ± 0.27	1.43 ± 0.7	0.26 ± 0.2
AMH (ng/mL)[a]	60.93 ± 30.39	1.31 ± 2.91	61.71 ± 32.03	1.76 ± 2.03	69.73 ± 31.29	1.52 ± 1.89
Inhibin B (pg/mL)[a]	209.83 ± 54.34	21.2 ± 15.71	186.8 ± 67.82	81.07 ± 85.28	214.89 ± 66.94	53.25 ± 47.25

25(OH)D, 25-hydroxyvitamin D; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, oestradiol; TT, total testosterone; AMH, anti-müllerian hormone.
[a]Statistically significant difference between groups in girls.

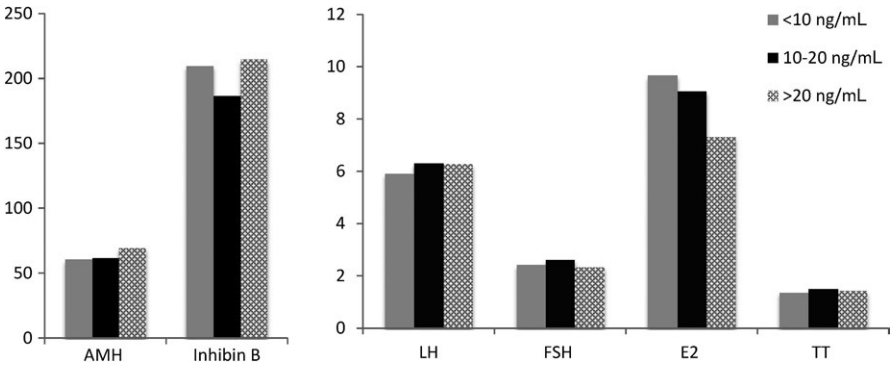

FIGURE 3 Hormone profile of boys with respect to 25(OH)D levels

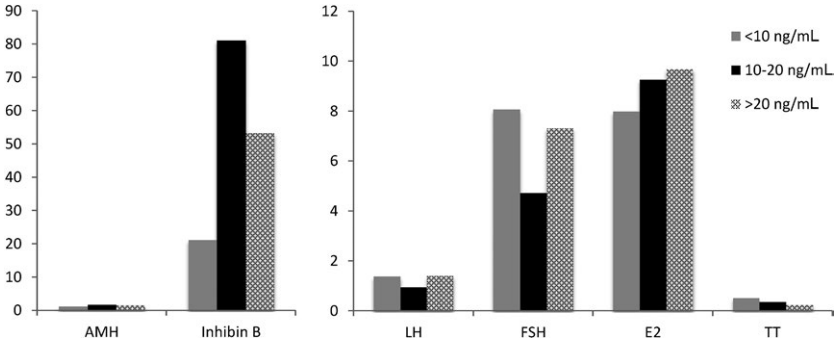

FIGURE 4 Hormone profile of girls with respect to 25(OH)D levels

inhibin B levels of the three groups ($P = 0.021$). <10 ng/mL vitamin D group showed a significantly low inhibin B levels compared to 10-20 and >20 ng/mL vitamin D groups ($P = 0.012$, $P = 0.02$). No statistically significant difference was detected between inhibin B levels of 10-20 and >20 ng/mL vitamin D groups ($P = 0.325$- Dunn's multiple comparison test). The correlation between vitamin D and LH, FSH and gonadal hormones in groups and hormone profile of girls with respect to 25(OH)D levels is shown in Table 3 and Figure 4.

4 | DISCUSSION

In this study, we found that almost 50% of infants had deficient/insufficient 25(OH)D levels despite replacement with 400 IU/d cholecalciferol from 3rd postnatal day onwards. This might emphasize the importance of vitamin D prophylaxis during infancy and questioning the compliance of the parents about vitamin D supplementation at each visit becomes highly important. Other than compliance issues, this finding may be due to technical problems in measurement of 25(OH)D concentrations. As it is well known, 25(OH)D measured currently consists of the sum of the 25(OH)D and 25(OH)D2 concentrations and is considered to be the best biomarker to define vitamin D status. Radioimmunoassay (RIA), high-performance liquid chromatography (HPLC) and electrochemiluminescence (ECLIA) are the methods used for the quantification of vitamin D (25OH)D in serum. Vitamin D metabolites that include vitamin D2 and D3 forms of 1α,25(OH)2D3, and 3-epi- 25(OH)D and 24,25(OH)2D3 as well as Vitamin D binding protein could affect the results.[20] Measuring parathyroid hormone

(PTH) levels would have overcome these technical issues in demonstrating real 25(OH)D since intact PTH suppression by serum 25(OH)D concentration has been used to estimate the 25(OH)D level to define hypovitaminosis D.

With respect to gonadal hormones, boys had higher LH and lower FSH levels which is consistent with the literature.[21] As expected, AMH, inhibin B and TT levels were higher in boys.[22] In contrast, E2 levels were not different in girls and boys. In their study, Ji et al[23] reported that serum E2 in female infants quickly fell to a minimum level at 1-2 months of postnatal life and sex difference disappeared. From this finding, they concluded that development of ovaries lags far behind the testis during infancy. In another study, Chellakooty et al[24] evaluated inhibin A, B, FSH, LH, E2 and sex hormone-binding globulin (SHBG) levels in 3-month-old girls and found overall low E2 concentrations, 15% being below detection limit, comparable with levels seen in early pubertal girls.

Epidemiological studies relating vitamin D to gonadal function in men give contradictory results. Some studies demonstrate a positive linear relationship between serum 25(OH)D concentrations and sperm motility.[25] Some other studies describe this relationship as being U-shaped meaning that both low and high levels of vitamin D negatively affect semen parameters.[26] The affects of vitamin D on reproductive capacity of men could be mediated directly through VDRs on testis, epididymis, prostate, and seminal vesicles. In addition, Sertoli cells have secretory activities which are ion channel-dependent and vitamin D has been shown to stimulate calcium uptake.[25] The affects could also be mediated through reproductive hormones. However, the data regarding the association between vitamin D levels and reproductive hormones are conflicting. There are studies reporting either a positive or no association between 25(OH)D concentration and reproductive hormones. Wehr et al[12] reported a higher TT and free androgen index (FAI) and lower SHBG concentrations in men with sufficient 25(OH)D levels than men with either insufficient or deficient 25(OH)D levels. In contrast, Hammoud et al[18] did not find any correlation between 25(OH)D and reproductive hormone values in their population, suggesting that the deleterious effect of high and low 25(OH)D on sperm parameters may not be mediated by reproductive hormones. Studies relating gonadotropins to vitamin D status were unable to find an association with the exception of one that found a tendency towards lower LH level in men with high 25(OH)D level.[14]

Inhibin B and AMH have been proposed as direct markers of Sertoli cell function and indirect markers of spermatogenesis. So, it is possible that vitamin D exerts some of its action on male reproductive system by affecting inhibin B and AMH levels. Blomberg et al[27] demonstrated a positive association between vitamin D status and inhibin B levels in infertile men. Similarly, a positive correlation has been reported between AMH and 25-OHD levels in men but not in 5- to 6-year-old boys.[28]

There is a critical period in human's life with respect to gonadal function called mini-puberty that is a process whereby pubertal hormones, FSH, LH, E2/testosterone in circulation, are increased as a result of activation of HPG axis.[19] Although biological significance of this period is still elusive, increase in the level of sex steroids in circulation during mini-puberty is thought to be important in the growth of external genitalia in infancy and testicular function in adulthood in male babies.[29] In female babies, it is important for the regulation of future ovarian function.[30] Thus, as in adulthood vitamin D could be an important relevant factor during this period of life with respect to gonadal function. However, in this study no association could be found between pituitary-gonadal hormones and vitamin D status in healthy boy infants at mini-puberty.

An important role has also been attributed to vitamin D in terms of female reproductive function. There is a strong evidence that vitamin D could be associated with polycystic ovary syndrome (PCOS), uterine leiomyomas, endometriosis and in vitro fertilization (IVF) outcomes.[31] Studies relating vitamin D to female hypothalamo-pituitary-gonadal hormone levels reveal contradictory results as in males. Females with PCOS are mostly studied and it has been stated that vitamin D deficiency could result in an imbalance in hyperandrogenism markers, such as serum dehydroepiandrosterone sulphate (DHEA-S), TT, FAI, free testosterone, and SHBG.[31] Yıldızhan et al reported a negative correlation between 25(OH)D and testosterone levels in obese patients with PCOS whereas Mishra et al did not find any correlation in between.[32,33] In our cohort of female babies at mini-puberty, we detected a significantly higher TT levels in vitamin D deficient group than sufficient group and a negative correlation between 25(OH)D and TT.

Anti-müllerian hormone and inhibin B secreted from granulosa cells of ovary are biomarkers of ovarian reserve in woman at reproductive age. A decrease in AMH and inhibin B together with an increase in FSH mark ovarian ageing. Researches on relationship of vitamin D with AMH, inhibin B and FSH give inconsistent results. In a recent study, no association was detected between 25(OH)D and biomarkers of ovarian reserve, namely AMH, inhibin B and FSH in a group of women aged 30 to 44 years.[34] Blomberg et al[35] demonstrated that vitamin D treatment increased serum inhibin B concentration and resulted in an insignificant increase in sperm production in infertile men with vitamin D deficiency.

In our study, we detected a lower inhibin B level in vitamin D deficient than sufficient girls at mini-puberty whereas no difference was present in AMH and FSH levels. In fact, FSH levels were higher and AMH levels were lower in vitamin D deficient girls but the difference did not reach to a statistically significant level.

As a conclusion, in this first study of relationship between reproductive hormones and 25(OH)D levels at mini-puberty, we were able to demonstrate a modest effect on inhibin B and TT levels in girls. Considering the importance of vitamin D on reproductive function in adults, we believe that randomized controlled trials, in which the effects of vitamin D supplementation during mini-puberty are analysed, are strongly needed and could clarify if there is a cause and effect relationship between vitamin D deficiency and gonadal hormone levels in early life.

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