Effect of Vitamin D Supplementation on the Salivary Cytokines of Pregnant Women: A Randomized Placebo-Controlled Trial

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Abstract

Objective: We aimed to determine the salivary cytokine profiles of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17 among pregnant women and determine if the cytokine profiles change upon administration of vitamin D to these women.

Methods: It was a community-based, blinded, placebo-controlled, randomized trial. The intervention group comprised pregnant females (n = 36) who received oral vitamin D 4000 IU/day for 6 months (starting from 12 - 16 weeks until the end of pregnancy). Controls comprised pregnant females who received placebo (n = 49) for the same duration. Probing depth (PD), attachment loss (AL) and bleeding on probing (BOP) were recorded. Saliva samples were subjected to multiplex ELISA analysis of cytokines. Serum vitamin D levels were determined. Outcome was assessed within 14 days post-partum. Periodontal examinations were done; blood and saliva samples were subjected to the set of tests described at baseline. SPSS 19.0 and Graph Pad 6.0 were used for analysis. The study was registered at www.clinicaltrials.gov with identifier number NCT01422122.

Results: The two study groups were comparable at the baseline. Although IL-6 showed wide variation, none of the pro-inflammatory cytokine levels was affected by six months of oral vitamin D supplementation. Similarly, the mean salivary levels of the anti-inflammatory cytokines IL-4 and IL-10 also remained unaffected in the two groups.

Conclusions: There was no association between hypovitaminosis D and periodontal disease among the studied sample of pregnant women. None of the salivary cytokines showed any significant change after six months of oral vitamin D supplementation to the pregnant women.

Keywords: Periodontal disease; pregnant women; saliva; cytokines; vitamin D

Introduction

Periodontal health is commonly affected in pregnancy (Löe and Silness, 1963). The prevalence of pregnancy gingivitis varies from 35% - 100%. Studies suggest that hypovitaminosis D is also a prevalent condition among pregnant women (Hollis and Pittard, 1984). Recent evidence suggests that there is an association between hypovitaminosis D and periodontal attachment loss (Grant and Boucher, 2010). This puts pregnant women with low levels of vitamin D at a greater risk of periodontal disease. In addition to serving as an essential micronutrient required for calcium balance (Holick, 2007) vitamin D possesses antimicrobial properties (Korf *et al.,* 2014) and has the ability to modulate immune response (Baeke *et al.,* 2010). The immunomodulatory property of vitamin D is routed via stimulation of antimicrobial polypeptides such as cathelicidins and defensins (Baeke *et al.,* 2010). Thus, it can be speculated that vitamin D supplementation to pregnant women (deficient in this micronutrient) will not only correct their deficiency but also likely to improve their periodontal health. It is imperative to note that the effect of vitamin D supplementation on the periodontal status in pregnant women has not been reported well in the literature.

Cytokines are the chemicals that mediate inflammatory processes in the body. Biochemically, these are soluble proteins produced by various cells that have vital immunological functions (Cekici *et al.,* 2014). Cytokines that have been reported in the periodontal literature are: interleukin-1β, C-reactive protein, interleukin-6, interleukin-8, TNF-α, MM-9, prostaglandin E_2, osteocalcin, and osteonectin, etc.

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Hence, these cytokines can potentially serve as biomarkers of periodontal inflammation (Garlet, 2010).

Cytokines such as IL-1β, IL-6, IL-17 and IFN-γ are known as pro-inflammatory cytokines because they have demonstrated a positive correlation with periodontal disease progression (Fu *et al*., 2013). Although there are some conflicting reports, collectively, these pro-inflammatory cytokines contribute to (or are differentially expressed in) periodontal tissue destruction (Okada and Murakami, 1998). However, few investigations have focused on the role of anti-inflammatory cytokines (mainly IL-4 and IL-10) in periodontal disease. These two cytokines have protective roles against alveolar bone breakdown, that is, reducing the periodontal disease progression (Liu *et al.,* 2010).

There is emerging evidence, using cytokine analysis, of profile changes serving as biomarkers of various health and disease states, but to date, there is just one meta-analysis published that has shown their utility in periodontal diseases (Stadler *et al*., 2016). It has been shown that salivary biomarker levels are not affected by mechanical periodontal treatment during pregnancy (Sexton *et al.,* 2011). Moreover, whether cytokine profiles change with administration of vitamin D supplementation in pregnancy is not known.

We aimed to determine the salivary cytokine profile (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17) among pregnant women and determined if the profile changes upon administration of oral vitamin D to these women. Lastly, we wanted to identify the best surrogate biomarker among the cytokines for prediction of periodontal disease during pregnancy.

Materials and methods

A community-based, double blind, placebo-controlled randomized trial was carried out in a rural area of Pind Dadan Khan in district Jhelum, Pakistan from September 2011 to August 2013. Pregnant females within 12-16 weeks of gestation (ultrasound confirmed) were inducted. Pregnant females with at least 20 natural teeth were included. Women with known high levels of vitamin D, diabetes, or presence of systemic disease, were excluded. Females who received any antibiotic treatment or dental prophylaxis in the previous 3 months were also excluded.

The laboratory phase was done at the Aga Khan University Hospital, Pakistan. A total of 86 pregnant participants were recruited. The written informed consents from the participants were obtained in the local language. The study was approved by the university ethics committee (ref# 147-Ped-ERC-2010) and was registered at the www.clinicaltrials.gov ID number NCT01422122.

The sample size was calculated using software (Sample Size Determination in Health Studies, WHO). To detect a 100 gram change in the birth weight anticipated with supplementation of vitamin D among pregnant, (mean birth weight 3100 g, SD: 150 g, difference: 100 g, level of significance: 0.05, power: 0.80), the required number was 36 per group. We inflated the required number by 20% to adjust for dropouts and hence inducted 86 pregnant females, assuming to get 43 expecting mothers in each arm of the study.

The study participants were randomized in blocks. The block randomization was originally made for another study (aimed at birth weight of newborns where > 400 pregnant women participated), but the present study focuses only on salivary cytokines. The present protocol was conceived, approved, and started at a later date; thus a big number of potentially eligible pregnant women had already passed their 16th week of pregnancy and consequently were not eligible for the present study. Furthermore, some eligible females in the randomized blocks did not consent to participate; therefore, the assumption of having equal numbers in the two study arms was not correct and they were later found to be distributed unequally. This was revealed at the end of the study when the randomization codes were opened.

We ended up with 36 participants in the intervention group and 49 in the placebo group. However, as mentioned earlier, the minimum required sample for this study was 36 subjects per group; therefore, the research question could still be addressed.

The study drugs (vitamin D and the placebo) were prepared by the university pharmacy services and packaged in the form of syrup. Both the vitamin D and placebo were supplied in identical bottles labeled either X or Y. The investigators, study staff, and the mothers were blinded to the group allocation. The allocation codes for vitamin D and placebo groups were kept in a sealed envelope in a locked cabinet in the university.

The intervention group comprised pregnant females (n = 36) who received an oral dose of 4000 IU of vitamin D daily for 6 months (starting from 12 - 16 weeks until the end of pregnancy). The control group comprised pregnant females who received placebo (n = 49) for the same duration.

The baseline dental examination for periodontal parameters was done by a trained dentist (FRK). The study participants were examined in daylight using sterilized dental examination instruments, while seated on a common house chair. A standard periodontal probe with Williams's markings was employed in recording the probing depth (PD), attachment loss (AL) and bleeding on probing (BOP). Probing depth and AL were obtained from six sites around each tooth, on six Ramfjord's teeth of all subjects, yielding a total of 36 sites in each subject. The distance was rounded down to the nearest whole millimeter for both measurements.

Study participants had at least one site with PD ≥ 3 mm, signifying periodontal disease. A sample of un-stimulated saliva was obtained (Navazesh, 1993) for multiplex ELISA analysis of cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17) at baseline. It was done on FACS array (BD-CBA Kit, BD Biosciences, San Jose, CA, USA). Vitamin D levels in serum were calculated (using DiaSorin-R kit, Stillwater, MN, USA).

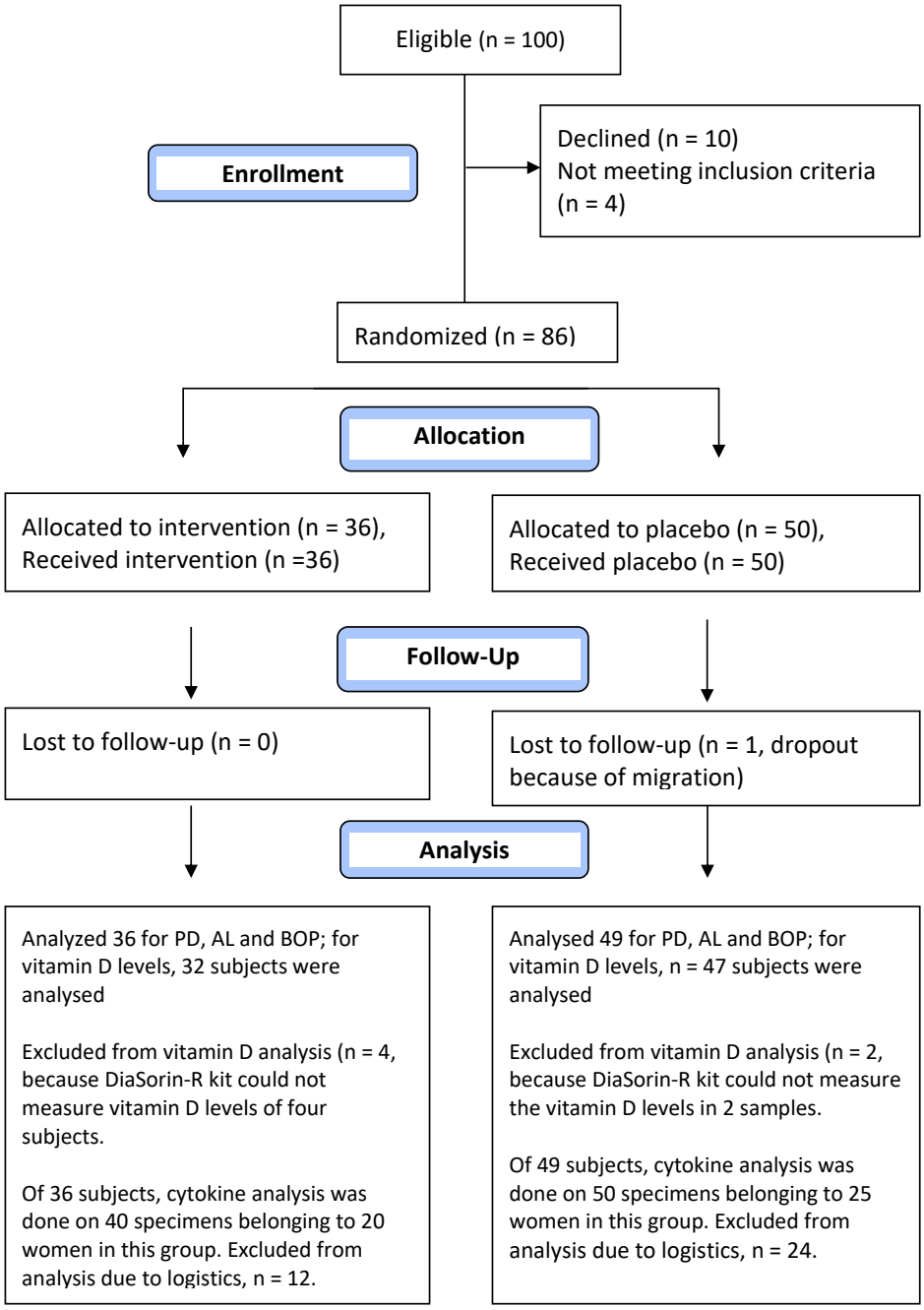

Figure 1. Study flow diagram as per CONSORT requirements. PD, probing depth; AL, attachment loss; BOP, bleeding on probing.

Outcome was assessed within 14 days of delivery (at least 20 weeks of vitamin D supplementation). Periodontal examinations was done and saliva samples were subjected to the multiplex ELISA as described for the baseline visit. The study flow diagram as per CONSORT requirements is shown as *Figure 1*.

Data analysis

Data were analyzed with SPSS 19.0 and figures were made with Graph Pad, Prism, version 6.0. Descriptive statistics, including mean and standard deviation (SD), of continuous variables were calculated while the frequency distribution of categorical variables was determined.

A paired *t*-test was applied for within group analysis (before-after comparison) of continuous variables such as probing depth and clinical attachment loss. The Chi-square test was used for difference in proportions for periodontal outcomes experienced in the study groups. Independent sample *t*-test was applied to compare the means of continuous variables between the intervention and placebo (age, BMI, PD, AL, and vitamin D). Repeated measures ANOVA were applied to see before-after difference in the vitamin D level, PD, AL and inflammatory biomarkers in the two study groups. The level of statistical significance for all tests was kept at 0.05.

Results

Among the pregnant women, 36 were given vitamin D supplementation and 49 received placebo. None of the participants ever received any dental implant or visited a dentist in the last 6 months. Similarly, none of the participants reported the use of dental floss. The participants were similar in age, body mass index, hemoglobin concentration, educational status and vitamin D levels at baseline. Dental and periodontal parameters such as number of teeth, plaque index, BOP, PD and AL were also similar in the two study groups at baseline. Vitamin D levels among pregnant women were alarmingly low. More than 80% pregnant women in the test group and nearly 90% in the placebo group were deficient in vitamin D, as shown in *Table 1*.

The mean vitamin D levels in the intervention and placebo at baseline were 12.9 ± 6.3 ng/mL and 12.7 ± 5.3 ng/mL, respectively. The vitamin D levels in the intervention group improved with supplementation but this improvement was not enough to reach a sufficient level (cutoff was 20.0 ng/mL). The placebo group showed a mild decline in the vitamin D level. This resulted in a statistically significant difference ($p < 0.01$) in the mean vitamin D levels at the endpoint. The PD and AL did show some improvement in the intervention group but the difference between the groups at endpoint remained statistically insignificant (*Table 2*).

Figure 2 demonstrates the pro-inflammatory cytokines (IL-2, IL-6, TNF-α, IFN-γ and IL-17) distribution in the two study groups at two time points. Although IL-6 showed wide variation, none of these pro-inflammatory cytokine levels were affected by vitamin D supplementation. Similarly, the mean salivary levels of the anti-inflammatory cytokines IL-4 and IL-10 also remained unaffected by six months of oral vitamin D supplementation in both groups (*Figure 3*).

Table 1. Characteristics of the study participants at baseline.

Variables	Intervention (n = 36)		Placebo (n = 49)		*p* - value
	Mean	SD	Mean	SD	
Age (years)	26.2	4.2	27.0	4.8	0.43
BMI	23.7	2.1	24.9	2.6	0.19
Hb (gm/dL)	8.6	1.4	8.9	2.1	0.18
No. of teeth	29.7	3.7	30.2	1.7	0.47
PD (mm)	1.8	0.5	1.8	0.6	0.87
AL (mm)	1.2	0.9	1.0	0.8	0.30
Vitamin D level (ng/mL)	12.9	6.3	12.74	5.3	0.90
	n	%	n	%	*p* - value
Vitamin D status					0.49
≤ 19.9 ng/mL	26	(81.2)	36	(89.2)	
> 20.0 ng/mL	6	(18.8)	4	(10.8)	
Parity status					0.31
0	10	(27.8)	11	(22.4)	
1 - 2	17	(47.2)	18	(36.7)	
≥ 3	9	(25.0)	20	(40.9)	
Educational status					0.16
≤ 5 years schooling	26	(72.2)	28	(57.1)	
≥ 6 years schooling	10	(27.8)	21	(42.9)	
Oral hygiene practice					0.89
Brush	24	(66.7)	32	(65.3)	
Miswak/dandasa	12	(33.3)	17	(34.7)	
DMFT scores					0.21
Low: 0 - 3	26	(72.2)	29	(59.2)	
High: ≥ 4	10	(27.8)	20	(40.8)	
BOP scores					> 0.99
Yes % sites	18	(50)	25	(51)	
No % sites	18	(50)	24	(49)	
Plaque index					0.87
0	12	(33.3)	18	(36.7)	
1	19	(52.8)	23	(46.9)	
2	5	(13.9)	8	(16.4)	

The differences between the groups were assessed using independent samples *t*-test or Chi-square (or Fisher exact) tests. Level of significance was kept at 0.05. BMI, body mass index; Hb, hemoglobin; PD, probing depth; AL, attachment loss; DMFT, decayed, missing, filled teeth; BOP, bleeding on probing.

Table 2. Comparison of two groups at the end-point for Vitamin D levels and periodontal parameters

Variable	Vitamin D (n = 36) Mean ± SD	Placebo (n = 49) Mean ± SD	*p* - value
Vitamin D levels in ng/mL	15.36 ± 7.61 (n = 32)	11.36 ± 4.71 (n = 40)	< 0.01*
Probing depth (PD) in mm	1.72 ± 0.52 (n = 36)	1.76 ± 0.60 (n = 49)	0.79
Attachment loss (AL) in mm	0.86 ± 0.80 (n = 36)	0.98 ± 0.91 (n = 49)	0.35
Plaque index	0.92 ± 0.67 (n = 36)	0.76 ± 0.60 (n = 49)	0.25

*Denotes statistical significance. Independent sample *t*-test was applied at $p \leq 0.05$. Vitamin D measurements were done in 72 participants only.

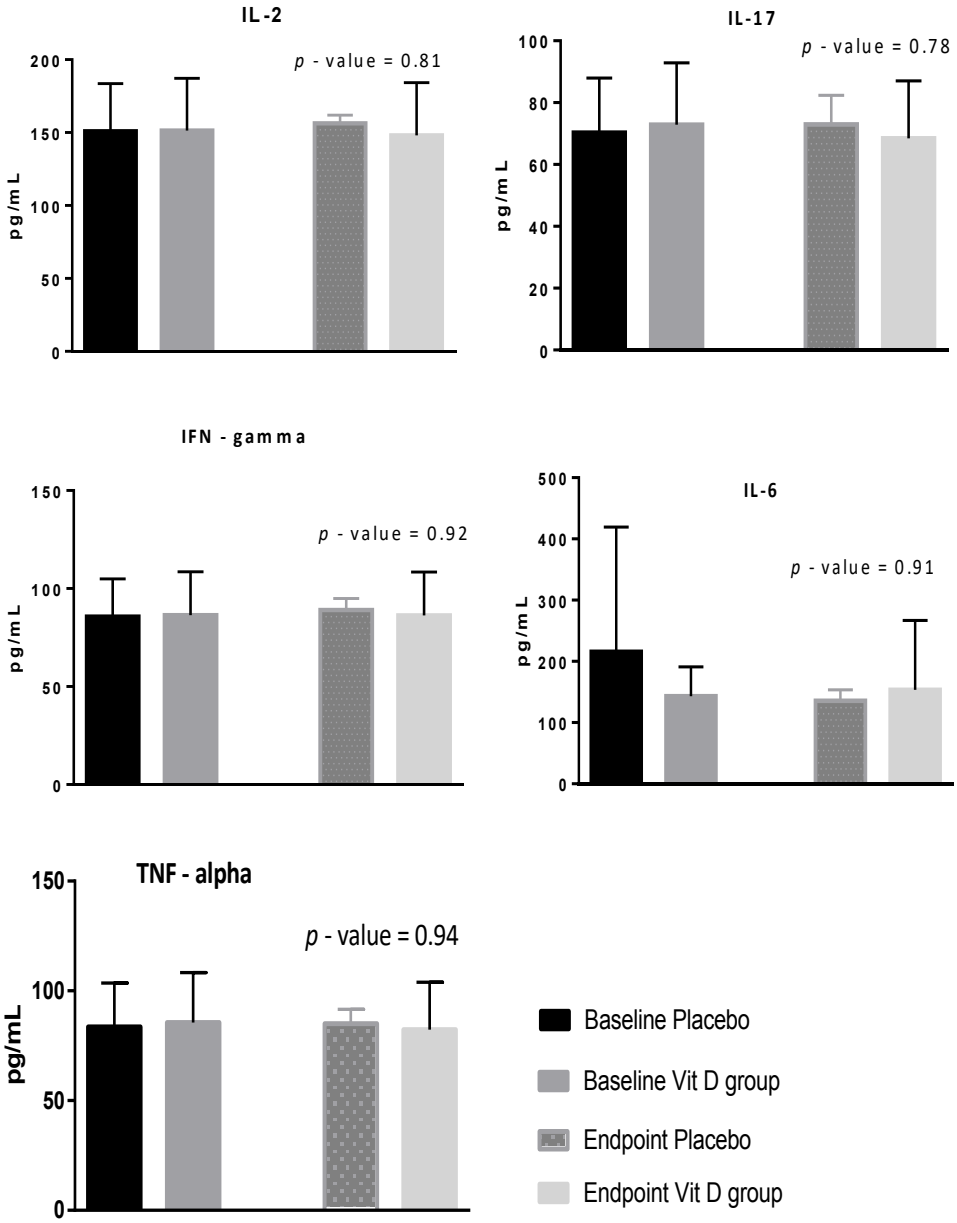

Figure 2. Pro-inflammatory salivary cytokines IL-2, IFN-γ, TNF-α, IL-17a and IL-6 in the two study groups at baseline and endpoint.

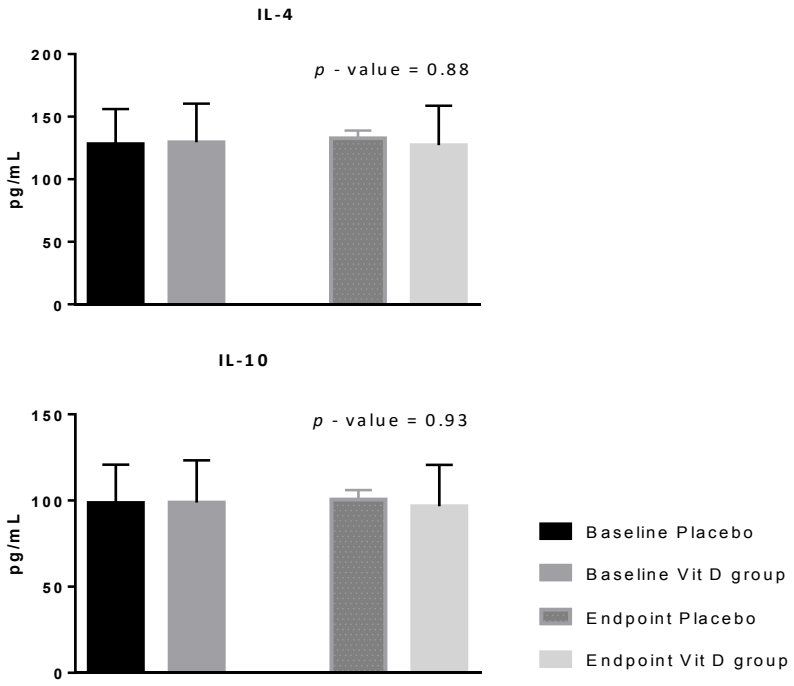

Figure 3. Levels of anti-inflammatory salivary cytokines IL-4 and IL-10 in the two study groups at baseline and endpoint.

Discussion

The present study is part of a larger trial that was planned to assess the effect of vitamin D supplementation on periodontal parameters, birth outcomes and salivary biomarkers associated with pregnancy. The detailed results of the periodontal parameters and birth outcomes have been published elsewhere (Khan *et al.,* 2016a; Khan *et al.,* 2016b). As part of community service, the trial subjects were offered free dental care as well (Khan *et al.,* 2015). However, the present report is confined to the salivary cytokines associated with the periodontal health among pregnant women and the changes exhibited in the cytokine profile after six months of vitamin D supplementation.

Although we included an array of seven cytokine biomarkers in the overall analysis, we found that they did not play any diagnostic role in identifying patients vulnerable to periodontal inflammation. This is in agreement with other reports that did not find any significance of cytokines in periodontal disease (Kinney *et al.,* 2011; Takahashi *et al.,* 1994; Becerik *et al.,* 2012). However, the only significant observation for these biomarkers in our study was an elevated expression of IL-6 among subjects exhibiting increased probing depth.

A study investigated the effect of periodontal treatment during pregnancy on the gingival crevicular fluid (GCF) and six cytokines associated with periodontal disease (Fiorini *et al.,* 2013). They observed a major reduction in periodontal inflammation among women subjected to periodontal therapy, with BOP declining from 50% to nearly 10%. Periodontal therapy significantly reduced the levels of IL-1β and IL-8 in crevicular fluid ($p < 0.001$). However, no significant effect of that therapy was observed on the serum cytokine levels. The postpartum levels of IL-1β in the GCF of the test group were also significantly lower than those of controls ($p < 0.001$). However, no significant differences between intervention and control for the serum cytokine levels were seen. In the present study, we used saliva instead of GCF, and probably that is why none of the cytokines correlated with periodontal parameters. It seems that salivary biomarkers are correlated more with the serum biomarkers than the GCF.

The main reason for using saliva in biomarker assessment is that it is easy and non-invasive to collect, and above all, it is readily available. Very few studies have longitudinally observed salivary biomarker profiles in patients with periodontal disease or have determined any longitudinal correlation with periodontal disease (Henskens *et al.,* 1996; Thomas *et al.,* 2009).

Salivary biomarkers such as MMP-8, OPG, MIP-1α, and IL-1β have associations with biological aspects of periodontitis and reflect improved periodontal health as a result of localized therapy over a 6-month period (Sexton *et al.,* 2011). For a pro-inflammatory biomarker to demonstrate clinical utility, its concentration should ideally reduce in response to the periodontal treatment. Conversely, anti-inflammatory biomarker levels should elevate after restoring periodontal health. We speculated that vitamin D supplementation to pregnant women would mimic the action of an adjuvant of periodontal therapy, and hence expected that salivary cytokine levels would respond. However, our results showed that salivary biomarkers among pregnant women are non-specific, and with the current data we are far from using them for clinical decision-making or incorporating them into the assessment of periodontal health or treatment planning.

The low levels of vitamin D in our sample of pregnant women can be attributed to the cultural and religious practice followed by women as mostly they keep their head, neck, and body covered (Khan *et al.,* 2016). This could restrict the amount of sunlight available to them. Moreover, increasing demands from the developing fetus, poor access to vitamin D-rich foods (such as fish) owing to socioeconomic constraints, and lastly genetic polymorphism in vitamin D receptors may have contributed to the widespread hypovitaminosis D in pregnancy (Anwar *et al.,* 2015).

The strength of the present study is that we used a randomized controlled trial design to identify the cytokines that could relate to periodontal disease and to identify whether vitamin D supplementation has any bearing on these cytokines. Moreover, the population we selected warrants special attention. Pregnant women in the rural part of a developing country like Pakistan, where access to maternal antenatal and dental services are limited, are more vulnerable to adverse outcomes.

There were a number of limitations in our study. We adhered to a rigid case definition of periodontal disease (PD > 3 mm), but still could not recruit more than 9 periodontitis participants. This is primarily because the prevalence of periodontal disease is low and sample selection was not based on the periodontal status of women. All the participants were otherwise healthy pregnant females. Secondly, we did not carry out a provisional analysis of vitamin D during the pregnancy.

Despite an oral vitamin D dose of 4000 IU per day for nearly 6 months, 92% of the women remained vitamin D-deficient. A likely explanation may be poor compliance among study participants, or possibly the drug had decomposed in the hot weather (as it was provided in a syrup form). Refrigerators were not available in the majority of households in the Pind Dadan Khan. We could not get the intervention drug and placebo made in the pill form.

Lack of an objective quantification of the periodontal attachment loss was another limitation. Ideally dental radiographs should have been employed, but with our limited logistics in the rural setting and the study subjects being pregnant women, it was precluded. Moreover, periodontal readings (PD and AL) were rounded to the nearest millimeter, resulting in loss of information.

Conclusions

There was no association between hypovitaminosis D and periodontal disease among the studied sample of pregnant women. None of the salivary cytokines showed any significant change with six months of oral vitamin D supplementation to the pregnant women. However, considering the limitations regarding the compliance of the participants with regard to vitamin D supplementation in the present study, further studies are needed to confirm the findings.

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