The impact of vitamin D on the innate immune response to uropathogenic \textit{Escherichia coli} during pregnancy

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Abstract

Urinary tract infections are highly common during pregnancy, and can cause serious complications for the mother and baby. Vitamin D, predominantly obtained from the sunlight, is known to have an effect on the urothelium, with immunomodulatory capacity against \textit{Escherichia coli} infection. However, its influence at this site remains to be further explored. This study therefore investigated its impact during pregnancy in a population of women who have the possibility of adequate year-round sun exposure. Serum from pregnant Ugandan women (\(n = 32\)) in each trimester of pregnancy, from women after delivery (\(n = 29\)) and from never-pregnant controls (\(n = 25\)) was collected. 25-Hydroxyvitamin D (25-OHD), cathelicidin LL-37, human β-defensin 2, interleukin (IL)-8 and soluble CD14 serum concentrations were measured by chemiluminescence immunoassay or ELISA. The ability of serum to inhibit \textit{E. coli} growth was tested. The immunomodulatory capacities of these serum samples and 1,25-dihydroxyvitamin D\textsubscript{3} were investigated in urothelial cells. Increases in 25-OHD and LL-37 levels were observed as pregnancy progressed, peaking in the third trimester. Serum 25-OHD levels were higher in multigravidae than in primigravidae, and correlated positively with maternal age. IL-8 levels were lower in the third trimester than in the first trimester, increased after delivery, but remained below those of never-pregnant women. Similarly, soluble CD14 concentrations increased after delivery. As gestation advanced, serum had an increased capacity to inhibit \textit{E. coli} growth. In vitro, it modulated the IL-8 response to infection in a vitamin D concentration-dependent manner. Our findings demonstrate that increasing vitamin D levels as pregnancy advances modulate the innate immune system towards a protective response to infection.

Keywords: 25-hydroxyvitamin D, \textit{Escherichia coli}, interleukin-8, LL-37, pregnancy, urinary tract infection

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Introduction

Urinary tract infections are the most commonly experienced infections in pregnant women \cite{1}, with \textit{Escherichia coli} being the main causative agent \cite{2}. Such infections may be associated with serious complications, including pre-term birth, intrauterine growth restriction, pre-eclampsia, and caesarean delivery \cite{3}. Therefore, treating these patients poses a significant challenge for clinicians \cite{1}.
Vitamin D is essential for human health, and is an important hormone in pregnancy. It is primarily produced when UVB radiation from the sun converts 7-dehydrocholesterol in the skin to vitamin D₃; this is followed by metabolic conversion to 25-hydroxyvitamin D (25-OH-D) and then to the active form, 1,25-dihydroxyvitamin D₃ (1,25D₃). It is also, to a lesser extent, absorbed from dietary sources such as fatty fish, dairy products, and fortified foods [4]. Vitamin D status is best reflected by serum 25-OH-D (S-25-OH-D) concentrations. Levels of <50 nmol/L are considered to be deficient, and those of ≥75 nmol/L are considered to be sufficient [5]. However, the minimal required and optimal 25-OH-D levels are still under debate, and the ideal levels during pregnancy have not yet been determined. Factors that reduce uptake of light from the sun, including dark, pigmented or covered skin and lack of vitamin D-containing dietary sources, may predispose to deficiency [4,6,7]. Low vitamin D levels during pregnancy may have long-term repercussions for the child [8].

Vitamin D plays a role in both adaptive and innate immunity. Its immunomodulatory capacity includes induction of antimicrobial peptides (AMPs) [9,10]. Notably, the promoter regions of the cathelicidin and human β-defensin (hBD) 2 genes contain consensus vitamin D response elements, allowing vitamin D to regulate their expression [11]. We have previously shown that, during *E. coli* infection, vitamin D regulates cathelicidin expression in the urinary tract [9]. Its effects in the human body are potentially far-ranging, as the vitamin D receptor is present on most immune cells [12], and the ability to modulate the expression of other immune molecules has been demonstrated [13].

Here, we sought to investigate the impact of vitamin D during pregnancy by exploring the vitamin D status of pregnant Ugandan women, and the serum concentrations of associated AMPs and other innate immunity markers. We also investigated the potential of serum to modulate the innate immune response in an in vitro model of urinary tract infection. Our findings overall indicate that vitamin D plays an important role in innate immune system preparation and protection during pregnancy.

**Study participants**

Pregnant women who attended Mulago Hospital Antenatal Clinic in Kampala, Uganda were recruited to this study. For 32 participants, serum samples were collected in the first, second and third trimesters. Sampling time-points were up to 13 weeks of gestational age, between 22 and 24 weeks, and between 32 and 34 weeks, respectively. In one group of women (n = 29), serum was collected both in the third trimester and 8–12 months after delivery. Exclusion criteria included the presence of multiple gestation, diabetes mellitus, any infection, including human immunodeficiency virus, or antibiotic use at the time of sampling. Only samples from women who were not newly pregnant and who still satisfied the original inclusion criteria were included for after-delivery samples. Samples from never-pregnant, age-matched women (n = 25) were collected as controls. Written informed consent was obtained from all participants. The study was approved by the Regional Ethics Committee in Stockholm, Sweden and Makerere University Faculty of Medicine Research and Ethics Committee, Mulago, Uganda.

**Analysis of samples**

Serum samples were protected from direct light exposure and were stored frozen at −80°C. All analyses were performed in Stockholm, Sweden. Serum 25-OH-D levels were measured by chemiluminescence immunoassay with the Liaison 25 OH Vitamin D Total Assay (DiaSorin, Saluggia, Italy). Concentrations of cathelicidin LL-37 (Hycult Biotech, Uden, The Netherlands), hBD2, interleukin (IL)-8 and soluble CD14 (sCD14) (R&D Systems Inc, Minneapolis, MN, USA) in serum and cell culture supernatants were measured by ELISA.

**Bacterial culture**

The uropathogenic *E. coli* (UPEC) strain CFT073 was cultured on blood agar at 37°C. Colonies were suspended in phosphate-buffered saline, and then centrifuged at 300 g for 10 min to remove bacterial aggregates. Concentrations were adjusted spectrophotometrically.

**Serum antimicrobial activity**

Serum antimicrobial activity was evaluated with sensitivity assays as previously described [14,15]. Briefly, UPEC CFT073 was cultured to mid-logarithmic phase in Luria–Bertani broth, and then embedded at a final concentration of 4.5 × 10⁵ CFU/mL into molten Luria–Bertani broth with 1% agar and low concentrations of electrolytes. After solidification in sterile Petri dishes, 3-mm-diameter holes were made, and 3 µL of serum was delivered. Following overnight incubation at 37°C, the diameter of the zone of inhibition was measured.

**Cell culture and infection assays**

The T24 human bladder epithelial cell line (ATCC HTB-4) and TERT-NHUC urothelial cells (provided by M. A. Knowles, Leeds, UK) were maintained in McCoy’s 5A medium with l-glutamine and 10% fetal bovine serum or EpiLife medium with 60 µM calcium and human keratinocyte growth supplement (Life Technologies, Carlsbad, CA, USA), respectively. Cultures were maintained at 37°C in a humidified incubator with 5% CO₂.
T24 cells were grown in 24-well cell culture plates (Costar) for 24 h. Then, the medium was exchanged with fresh McCoy’s 5A medium supplemented with 10% serum from Ugandan women in the third trimester of pregnancy who had low (<50 nmol/L) or high (>80 nmol/L) S-25-OHD levels. After 24 h, cells were infected with UPEC CFT073 (1 × 10^6 CFU/mL) and treated with gentamicin (40 μg/mL) for 24 h.

TERT-NHUC cells grown overnight in Primaria 24-well plates (BD, Franklin Lakes, NJ, USA), and then treated for 24 h with 1,25D3 and gentamicin (40 μg/mL). An ethanol vector control was maintained alongside. Cells were then infected with UPEC CFT073 (1 × 10^6 CFU/mL) for 4 h. For all assays, medium was collected and centrifuged at 300 g for 10 min, and the supernatants were stored at -80°C pending ELISA testing.

**Total RNA extraction and real-time RT-PCR**

Total RNA was extracted from cells with the RNeasy Mini kit (Qiagen, Hilden Germany); 1 μg was transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturers’ instructions. Gene expression was analysed with the TaqMan Gene Expression Assay for IL-8 (Hs00174103_m1). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control to calculate relative gene expression.

**Statistical analysis**

Serum concentrations are presented as mean ± standard deviation or median and range. Data were analysed with parametric or non-parametric repeated measures ANOVAs or paired or unpaired t-tests, as appropriate. For in vitro assays, unpaired t-tests or one-way ANOVAs with Bonferroni’s post hoc test were used. Pearson’s r-test was used for correlation between S-25-OHD levels in serum and maternal age. Statistical analyses were performed with GraphPad Prism Version 5.04. A p-value of <0.05 was considered to be significant.

**Results**

**Participant characteristics**

Pregnant women were aged 18–31 years, with a median 23 years. Never-pregnant controls were aged 20–25 years, with a median of 23 years. No women were supplemented with vitamin D. Only one participant reported consuming fortified food products before the collection of serum.

**S-25-OHD and cathelicidin LL-37 levels increase as gestation advances and decrease after delivery**

Over the course of pregnancy, S-25-OHD deficiency (≤50 nmol/L) was similarly prevalent at 25%, 22% and 25% in the first, second and third trimesters, respectively. After delivery, 31% of participants (n = 29) had deficient levels, as compared with 36% of never-pregnant women (n = 25). However, S-25-OHD levels increased significantly (p < 0.01) over the course of pregnancy, with higher levels being seen in the second and third trimesters (Fig. 1(a)). Individual S-25-OHD levels tended to decrease between the third trimester and after delivery (68.6 ± 25 nmol/L vs. 62.7 ± 20.7 nmol/L, respectively), although the difference was not statistically significant (p = 0.08).

S-25-OHD levels were significantly (p ≤ 0.01) lower in primigravidae (n = 8) than in multigravidae (n = 24) (Fig. 1(b)). Primigravidae tended to be younger (22 ± 1.7 years vs. 24.5 ± 3.4 years). Overall, there was a positive correlation between S-25-OHD levels and age (r 0.36, p < 0.05) in each trimester of pregnancy.

Similarly to S-25-OHD levels, serum LL-37 levels increased as pregnancy progressed (Fig. 1(c)). They were significantly higher in the third trimester (n = 16, 77 ± 17.7 pg/mL) than after delivery in the same women (60.8 ± 17.1 pg/mL, p 0.012). This indicates that LL-37 might be of importance leading up to and during delivery. Serum hBD2 levels were similar in the first trimester (n = 18, 185.5 ± 178.2 pg/mL), in the third trimester (n = 18, 196.8 ± 149.3 pg/mL), after delivery (n = 10, 174.9 ± 106.7 pg/mL), and in never-pregnant controls (n = 8, 164.5 ± 67.6 pg/mL).

**Serum antimicrobial capacity increases as pregnancy progresses**

Increasing antimicrobial capacity over the course of pregnancy was observed, in line with changes seen in serum LL-37 levels (Fig. 1(d)). Interestingly, however, samples from never-pregnant women inhibited E. coli growth significantly (p < 0.01) more than first-trimester, second-trimester and after-delivery samples (Fig. 1(d)).

**Serum IL-8 (S-IL-8) and sCD14 levels are lower during pregnancy than after delivery**

High S-IL-8 levels have been identified as a marker of preterm labour [16]. We observed significantly lower S-IL-8 levels in the third trimester than in the first trimester, after delivery, and in never-pregnant controls (p < 0.05; Fig. 2(a)). Levels after delivery remained low in comparison with never-pregnant controls, but were higher than in the third trimester. This indicates that, at certain periods during pregnancy and after delivery, suppression of IL-8 is important.

We then investigated CD14, a key co-receptor in the host defence against pathogens [17]. Analysis of samples collected from women (n = 18) in the first and third trimesters revealed no significant difference (1.3 × 10^6 ± 2.3 × 10^5 pg/mL vs. 1.3 × 10^6 ± 2.5 × 10^5 pg/mL, respectively). Interestingly,
however, in women from whom an after-delivery sample was collected \((n = 10)\), third-trimester sCD14 levels were significantly lower than after delivery \((1.3 \times 10^6 \pm 2.7 \times 10^5 \text{ pg/mL} \text{ vs.} \ 1.6 \times 10^6 \pm 3.8 \times 10^5 \text{ pg/mL}, \text{ respectively, } p < 0.05)\).

**The urothelial IL-8 response to UPEC is reduced by vitamin D**

We next explored the potential of serum from pregnant women with different 25-OHD levels to modulate the IL-8 response to UPEC. The urothelial IL-8 response to infection was significantly less in cells treated with serum containing high 25-OHD levels \((>80 \text{ nmol/L})\) than in those treated with low 25-OHD levels \((<50 \text{ nmol/L}, p < 0.05)\) (Figs. 2(b) and (c)). This indicates that adequately high vitamin D concentrations in the serum are protective against infection. We could not exclude the possibility that other serum components may have influenced these findings, so we next verified the vitamin D-specific role in this immune response. In vitro results showed that the degree of IL-8 induction was \(1.25D_3\) dose-dependent (Fig. 2(d) and (e)). At a concentration of \(10^{-8}\) M, \(1.25D_3\) significantly...
reduced IL-8 induction in both non-infected and UPEC-infected cells in comparison with non-treated cells (p < 0.05; Fig. 2(e)); a less pronounced effect was seen with a lower vitamin D concentration.

No vitamin D concentration-dependent differences in CD14 mRNA or sCD14 protein expression were observed in serum-treated T24 cells (data not shown), and hBD2 and LL-37 mRNA expression was not detected.

**Discussion**

We here demonstrate that, in pregnancy, S-25-OHD and serum LL-37 levels increase, S-IL-8 levels decrease and the serum from pregnant women has a higher capacity to inhibit UPEC growth as gestation advances. After delivery, S-25-OHD and serum LL-37 levels decrease as S-IL-8 and serum sCD14 levels increase. We show the immunomodulatory capacity of serum and vitamin D through downregulation of urothelial IL-8 expression during infection.

Uganda is located on the equator between latitudes 4°N and 2°S, and experiences tropical and mostly sunny weather; under these conditions, it is expected that there will be adequate exposure to UV light for optimal synthesis of vitamin D, including in people with dark skin of Fitzpatrick type VI [18]. The combination of dark, pigmented skin and lack of vitamin D-containing foods in the diet probably contributed to the low S-25-OHD levels seen in study participants. Fortified foods are...
rare in Uganda, and only few study participants reported consuming foods containing naturally occurring vitamin D. Although some authors have reported similar vitamin D findings to ours [19,20], decreasing levels over the course of pregnancy have also been described [21]. These contradictory findings may be attributable to diet, cultural practices related to pregnancy and sun exposure, genetic differences, or differences in the method used to measure S-25-OHD. The role of vitamin D-binding protein (DBP) may also be considered, as vitamin D circulates mostly bound to DBP. Differences in DBP levels and affinity in different communities with polymorphisms in the DBP gene have been associated with low DBP levels [22].

To the best of our knowledge, this is the first time that LL-37 and hBD2 serum levels have been profiled during pregnancy. Our finding of increased LL-37 levels as gestation advances probably reflects progressive immune system strengthening leading up to delivery. In view of the important role of LL-37 in the urinary tract [15], the respiratory tract [10], and elsewhere [23], elevated LL-37 levels probably confer increased immune protection against such infections. Higher levels at later stages of pregnancy would also benefit the neonate, as cord plasma LL-37 levels at delivery have been correlated with maternal plasma levels [24]. Our finding of progressively more effective inhibition of E. coli growth further supports our hypothesis. In the absence of a statistically significant relationship with serum LL-37, however, we believe that our findings mirror a synergistic effect of several AMPs in serum [25].

Serum from pregnant women has low IL-8 levels, and we additionally show that it has the immune potential to down-regulate the proinflammatory IL-8 response to UPEC infection. Our data indicate the role of vitamin D in modulating this response, supporting previous findings that vitamin D has anti-inflammatory functions [26]. In the context of pregnancy, such an anti-inflammatory effect would be beneficial, as a successful pregnancy requires fine-balanced immunomodulation, while proinflammatory processes are associated with labour [27].

In line with changes in S-IL-8 levels, we here demonstrate higher serum sCD14 levels after delivery. Whereas we were able to detect CD14 mRNA expression in T24 cells, contradictory findings have previously been reported in these cells [17,28]; differences in cell culture practice may underlie this [28]. Furthermore, we did not find a serum vitamin D concentration-dependent effect on its expression during infection. We thus support the idea that already circulating sCD14 is important for recognition of bacterial lipopolysaccharide in the urothelium [17].

We found vitamin D deficiency to be common among both pregnant and non-pregnant Ugandan women, despite the year-round possibility of sun exposure. Interestingly, however, older pregnant women had higher levels than younger women, a finding in line with those of others [29]. Recent recommendations suggest that intake of up to 2000 IU/day of cholecalciferol may be required to achieve sufficient S-25-OHD levels during pregnancy [30]. The impact of maternal vitamin D deficiency cannot be underestimated, as the fetus depends on 25-OHD crossing through the placenta, and breast milk is an important source of 25-OHD after birth [31]. The increased risk of hypocalcaemia and rickets in the baby [32] indicate that supplementation would be valuable for this or any other patient group at risk of deficiency.

Our results demonstrate that, even in locations where there is adequate access to sunlight, there is a risk of vitamin D deficiency during pregnancy. In view of our findings, we suggest that there may be a need for supplementation of pregnant women who have deficient S-25-OHD levels. This would have the potential to alter the immune responses against infection away from potentially detrimental inflammatory processes and more towards protective antimicrobial activity.

**Transparency declaration**

The authors declare that they have no conflicts of interest.

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**References**
