Vitamin D supplementation during pregnancy inhibits the activation of fetal membrane NF-κB pathway

N. GURKAN

Department of Obstetrics and Gynecology, Medical Park Hospital, Samsun, Turkey

Abstract. - OBJECTIVE: Nuclear Factor-κB (NF-κB) is an important member of the basic cellular inflammatory pathway that regulates inflammation and apoptosis in fetal membranes. Vitamin D (VD) exerts its anti-inflammatory and immunomodulatory effects via the NF-κB pathway. This study was designed to investigate amniotic fluid NF-κB levels in pregnant women undergoing VD replacement therapy.

PATIENTS AND METHODS: Sixty patients who received antenatal vitamin D supplementation from the 14th week of pregnancy until delivery were included in the study. Participants were selected among those whose serum vitamin D levels were compatible with insufficiency (20-30 ng/mL), according to the Endocrine Society proposal. Participants were divided into three groups with 20 patients in each group and one of the cholecalciferol or placebo treatments was given. Patients in Group 1 were given 500 IU/day of cholecalciferol, while patients in Group 2 were given 1000 IU/day of cholecalciferol. Patients in Group 3 were given cholecalciferol treatment (placebo). Patients in all groups underwent elective cesarean section. Amniotic fluid samples were collected after the fetal membranes were removed and before the fetal parts were manually removed.

RESULTS: The amniotic fluid NF-κB level of the control group who did not receive VD replacement was 9.33±2.02 ng/mL. The amniotic fluid NF-κB level of the 500 IU/day VD replacement group was found to be 6.12±1.23 ng/mL. Compared to the control group, NF-κB levels of pregnant women given 500 IU/day VD replacement were significantly lower (9.33±2.02 ng/mL vs. 6.12±1.23 ng/mL, p<0.03). The amniotic fluid NF-κB level in the 1000 IU/day VD replacement group was found to be 3.09±0.44 ng/mL. Compared to the control group, amniotic fluid NF-κB levels of pregnant women given 1000 IU/day VD replacement were significantly lower (9.33±2.02 ng/mL vs. 3.09±0.44 ng/mL, p<0.01). When the VD replacement groups were compared among themselves, the amniotic fluid NF-κB level decreased approximately twice as much in the 1000 IU/day replacement group compared to the 500 IU/day replacement group (3.09±0.44 ng/mL vs. 6.12±1.23 ng/mL, p<0.01). A negative correlation was found between amniotic fluid NF-κB level and VD dose (r=-0.789, p<0.04).

CONCLUSIONS: The present study showed for the first time that amniotic fluid NF-κB levels decreased in pregnant women who underwent VD replacement dose dependent manner.

Key Words: Pregnancy, Vitamin D, Amniotic fluid, NF-κB.

Introduction

Although the changes caused by pregnancy in vitamin D (VD) metabolism are not known exactly, biochemically low VD levels are frequently encountered during pregnancy1,2. Deficiency in dietary intake of ergocalciferol or cholecalciferol or increased skin pigmentation due to pregnancy may lead to a decrease in VD levels3. On the other hand, the increased need for calcium and phosphate during pregnancy due to the fetus and its appendages may also lead to a decrease in VD levels. As it is known, the fetus is completely dependent on the mother in terms of VD. Therefore, maternal and umbilical cord VD levels are highly correlated4. The VD passes through the placenta and reaches the fetus and its appendages5. While maternal serum ionised calcium levels are kept stable during pregnancy, active 1,25(OH)2 D levels are increased5. Trying to keep calcium and phosphate metabolism stable and increasing 1,25(OH)2 D during pregnancy are important in providing immune tolerance to the fetus6. VD replacement in pregnant women increases umbilical cord VD levels as well as neonatal serum VD levels7. In addition to providing bone mineral density and muscle development of the fetus, VD also plays an important role in the continuation of the strong immunomodulatory effect mediated by T and B cells and the prevention of
inflammatory reactions. Chorioamnionitis with antenatal inflammation, which is thought to develop in VD deficiency, may lead to the emergence of different pathophysiological processes. In VD deficiency, the fetus becomes more prone to many acute and chronic inflammatory diseases especially respiratory system and fetal membranes. It has been reported that low VD induces premature rupture of membranes and uterine contractions through chorioamnionitis and may lead to preterm delivery. However, the researchers reached this conclusion with observational studies and did not investigate the inflammatory changes that are claimed to occur in the fetal membranes.

Nuclear Factor-κB (NF-κB) is an important member of the basic cellular inflammatory pathway that regulates inflammation and apoptosis. NF-κB is inactive in the cytosol by complexing with inhibitor κB. VD regulates its anti-inflammatory and immunomodulatory effects via T and B cells as well as NF-κB pathway. Thinning and tearing of fetal membranes is a multifactorial regulated process. In addition to hormonal factors and uterine contractions, inflammation of the chorioamniotic membranes also plays an important role in the initiation of labor. Decreased tensile strength and rupture of fetal membranes in VD deficiency may be regulated via the NF-κB pathway. The reason for preterm rupture of membranes in VD deficiency may be NF-κB-dependent.

Materials and Methods

Patient Selection and Grouping

Patients who received antenatal vitamin D replacement from the 14th week of pregnancy until delivery were included in the study. Participants were selected among those whose serum vitamin D levels were compatible with insufficiency (20-30 ng/mL), according to the Endocrine Society proposal. Sixty pregnant women were divided into three groups with 20 patients in each group and one of the cholecalciferol or placebo treatments was given. Patients in Group 1 were given 500 IU/day of cholecalciferol, while patients in Group 2 were given 1000 IU/day of cholecalciferol. Cholecalciferol doses were determined according to the Maternal Vitamin D Osteoporosis Study (MAVI-DOS). The patients in group 3 were given placebo (cholecalciferol treatment). All the patients were included in the study until delivery. Missed doses were administered up to 7 days. Preparations containing calcium, phosphorus, iron, and vitamin D were always provided to patients during vitamin D treatment.

Inclusion criteria were determined as follows. Women aged 20 years and older with spontaneous pregnancy and 14 completed weeks of gestation according to USG or last menstrual period (LMP). Patients with more than one USG record, the first record was considered. Accordingly, we considered 14 weeks + 0 days to be included in the study. Estimated date of birth was determined according to USG or last menstrual period (LMP). Estimated date of birth was determined according to second trimester USG. Those with a history of hypertension, VD, hypercalcemia, or kidney stones, as well as those with active tuberculosis, parathyroid pathology, liver or kidney disease, hypertension or proteinuria were excluded from the study. Multiple gestations, IVF/ICSI pregnancies, oligohydramnios and epilepsy drug users were also excluded from the study. Patients who planned for normal vaginal delivery were not included in the study because it was not suitable for the study design. Those with a history of PPROM, patients with placenta previa or ablatio placenta, patients with a history of diabetes mellitus or gestational diabetes mellitus, and those with a C/S decision due to preeclampsia and eclampsia were not included in the study. Since the cervical softening mechanism is different in breech presentations, these patients were excluded from the evaluation.

Patients in all three groups underwent elective cesarean section. Amniotic fluid samples were collected after the fetal membranes were cut and before the fetal parts were manually removed. Sterile 10 cc injectors were used for amniotic fluid collection. Care was taken not to contaminate the samples with blood. Amniotic fluid samples containing dense blood and vernix caseosa were not included in the study. Samples containing mild to moderate blood or vernix were included in the study after centrifugation. The primary
outcome was to determine the amniotic fluid NF-κB levels of pregnant women who received and did not receive VD. The secondary outcome was to correlate the amniotic fluid NF-κB with the dose of vitamin D administered and other laboratory, demographic parameters. All procedures performed in this study were in accordance with the Ethical Standards of International Research Committee and local approval was obtained from the OMU. All patients recruited to the study were fully counseled and written informed consent was obtained.

### NF-κB Analysis in Amniotic Fluid
NF-κB levels were measured using ELISA after thawing the amniotic fluid samples of all groups that were taken during cesarean section and frozen in RNA later. The immunological kit used can measure NF-κB levels in biological fluids with great sensitivity (CusabioBiotechCo., Ltd., WUHAN, CHINA). The kit can make in vitro and quantitative measurements in humans. Thawed amniotic fluids were treated with phosphate buffer and centrifuged at 2500 rpm for 5 minutes. Thus, blood and vernix were removed. NF-κB was analyzed according to the method specified by the manufacturer in the kit. Results are given in ng/mL. The detection range of the kit was 0.3 to 20 ng/mL and the minimum measurable level was 0.078 ng/mL. The intra- and inter-assay coefficients of variation of the kit were <8% and <10%, respectively. Test results are expressed as ng/mL.

### Statistical Analysis
The data obtained following VD replacement was analyzed with the use of the Statistical Package for Social Sciences software 21.0 for windows package software (SPSS Inc., Armonk, NY, USA). Normality of data was examined by nonparametric Kolmogorov–Smirnov test. Continuous variables were analyzed using parametric One-Way ANOVA test. Pearson's correlation analysis was used to determine the correlation between amniotic fluid NF-κB levels and other parameters. Data are presented as mean ± SD. A *p* value <0.05 was considered statistically significant.

### Results
Demographic, laboratory, and perinatal characteristics of patients who did and without VD replacement are presented in Table I. There was no significant difference between the groups in terms of serum VD, age, gravidity, parity, and gestational age. The VD levels of all experimental and placebo groups were determined as 20-30 ng/mL, consistent with insufficiency. Post-replacement VD levels were not evaluated. There was no difference between the groups in terms of fetal birthweight and elective cesarean delivery was performed without complications in all groups.

The amniotic fluid NF-κB level of the control group who did not receive VD replacement was found to be 9.33±2.02 ng/mL. The amniotic fluid NF-κB level of the 500 IU/day VD replacement group was found to be 6.12±1.23 ng/mL. Compared to the control group, NF-κB levels of pregnant women given 500 IU/day VD replacement were significantly lower (9.33±2.02 ng/mL vs. 6.12±1.23 ng/mL, *p*<0.03). The amniotic fluid NF-κB level of the 1000 IU/day VD replacement group was found to be 3.09±0.44 ng/mL. Compared to the control group, amniotic fluid NF-κB levels of pregnant women given 1000 IU/day VD replacement were significantly lower than the controls.

<table>
<thead>
<tr>
<th>Group 1 (n=20) 500IU/day Vitamin D</th>
<th>Group 2 (n=20) 1000 IU/day Vitamin D</th>
<th>Group 3 (n=20) Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.9±6.10</td>
<td>29.1±5.30</td>
</tr>
<tr>
<td>VD level (ng/mL)</td>
<td>24.4±4.51</td>
<td>25.9±6.03</td>
</tr>
<tr>
<td>Gravida</td>
<td>3.20±1.02</td>
<td>2.90±1.44</td>
</tr>
<tr>
<td>Parity</td>
<td>2.11±0.33</td>
<td>1.87±0.40</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>37.2±8.33</td>
<td>36.6±5.31</td>
</tr>
<tr>
<td>Fetal birthweight (gr)</td>
<td>2895.1±149.2</td>
<td>2945.4±202.1</td>
</tr>
<tr>
<td>Amniotic fluid NF-κB (ng/mL)**</td>
<td>6.12±1.23</td>
<td>3.09±0.44</td>
</tr>
</tbody>
</table>

*Estimated date of birth was determined according to LMP or USG. **Amniotic fluid NF-κB levels of both groups who received 500 or 1000 IU/day VD replacement were found to be significantly lower than the controls.*
Amniotic fluid NF-κB and vitamin D

Amniotic fluid NF-κB and vitamin D parameters and NF-κB levels. A negative correlation was found between amniotic fluid NF-κB level and VD dose (r=-0.789, p<0.04). No significant correlation was found between other maternal and perinatal parameters and NF-κB levels.

Discussion

There is no consensus on both the definition of VD deficiency in pregnancy and the dose of VD to be used for supplementation. The threshold values used for the definition of deficiency vary from country to country. In this study, we selected the patient groups among pregnant women with VD insufficiency (25-35 ng/mL). In general, the recommended dose for VD support during pregnancy varies between 400-600 IU/day. However, the World Health Organization has not determined a specific dose for VD support during pregnancy22. On the other hand, while the Endocrine Society determines the upper limit of the maximum safe VD dose as 10,000 IU/day, the World Health Organization criteria. We showed that VD at a dose of 500 IU/day significantly reduced amniotic fluid NF-κB levels. The inhibitory effect of VD on the fetal membrane NF-κB pathway via the VD receptor (VDR). In the same placental inflammation model, Vit D administration has been shown to inhibit both placental NF-κB signaling and VD receptor expression. In the same study, it was emphasized that VD exerts anti-inflammatory effect in placental tissue via VDR and VD receptors24. The mechanisms that initiate labor are not fully known, local and systemic hormones, inflammatory cytokines whose synthesis and release in fetal membranes as a result of the synchronized activation of fetal adrenal cortex, maternal brain membranes may also be involved. While cascading local and systemic inflammatory factors cause inflammation of the membranes, matrix metalloproteinases cause the inflammatory changes to rupture and stimulate the release of labor. NF-κB may play a role in this reaction cascade as the main regulator of inflammation. The release of adhesion molecules such as E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 is regulated by the NF-κB pathway23. VD may contribute to the regulation of inflammatory pathways in fetal membranes with its immunomodulatory and anti-inflammatory effects. In good agreement with this in the LPS-induced placental inflammation model, Vit D administration has been shown to inhibit both placental NF-κB signaling and VD receptor expression. In the same study, it was emphasized that VD exerts anti-inflammatory effect in placental tissue via VDR and VD receptors24.
a similar mechanism\textsuperscript{24,28,29}. The inhibition of the decidual cells differentiation by 1,25(OH)\textsubscript{2}D\textsubscript{3}\textsuperscript{30,31} may explain the reduction of NF-\kappaB synthesis in fetal membranes by VD. The increased synthesis of pro-inflammatory cytokines in VDR knock-out mice\textsuperscript{32} is an important proof that the NF-\kappaB-blocking effect of VD is mediated by VD receptors. Moreover, in line with our results, another strong evidence is that 1,25(OH)\textsubscript{2}D\textsubscript{3} blocks cytokine release in uNK cells\textsuperscript{33}.

VD inhibits the NF-\kappaB pathway through different mechanisms in different cells. While it blocks the binding of NF-\kappaB to DNA in some cells, it decreases RelB and VD receptor expression in others and sometimes prevents the nuclear translocation of p65\textsuperscript{34-36}. On the other hand, it is not known exactly how VD blocks NF-\kappaB in fetal membranes. VD replacement may increase the synthesis of many local and systemic factors that block NF-\kappaB translocation to the nucleus\textsuperscript{37-39}. Since the patients were sent to elective cesarean section we could not establish a correlation between the delivery times of the VD reperation groups and NF-\kappaB levels. Since active labor can affect NF-\kappaB levels by stimulating inflammation in fetal membranes, we had to design the study in this way.

Conclusions

We showed for the first time that amniotic fluid NF-\kappaB levels decreased in pregnant women who underwent VD replacement, which was statistically important in terms of blocking the role of the NF-\kappaB pathway in the initiation of labor. In addition, this may open new horizons in the treatment of obstetric emergencies such as preterm labor and premature rupture of fetal membranes due to the inhibitory effect of VD on the placental NF-\kappaB signaling pathway\textsuperscript{24}.

Conflicts of Interest

The authors declare no conflicts of interest.

Ethical Committee

Ethical Standards of International Research Committee and local approval was obtained from the OMU.

Informed Consent

All patients recruited to the study were fully counseled and written informed consent was obtained.

ORCID

N. Gurkan 0000-0003-1088-018X.

References


