Preventive Effects of Vitamin D on Seasonal Influenza A in Infants: A Multicenter, Randomized, Open, Controlled Clinical Trial

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L.T.H., J.Z., J.D., Y.C.W., and Y.M.S. performed the experiments; L.T.H., H.L.L., Y.C.W., and
J.D. analyzed the data; J.Z. and L.T.H. contributed reagents/materials/analysis tools; and J.Z.
wrote the paper.

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Abstract

Objectives: This study aimed to evaluate the clinical efficacy and safety of vitamin D for preventing influenza A in 400 infants in a multicenter, randomized, open, controlled clinical trial.

Methods: The infants were randomized into low-dose and high-dose vitamin D groups, and serum calcium, inorganic phosphorus and 25-hydroxyvitamin D levels were detected thrice in 4 months. Infants infected with influenza A were monitored for symptoms including fever, cough, and wheezing. Pathogen levels and safety of vitamin D treatment were also evaluated.

Results: Of 121 cases in total, 78 and 43 cases of influenza A infection occurred in the low-dose and high-dose vitamin D groups, respectively. There was a significant difference between the groups ($\chi^2 = 14.6324$, $P = 0.0001$). Among the cases of influenza infection, the median durations for fever, cough, and wheezing were shorter in the high-dose vitamin D group than in the low-dose vitamin D group. The viral loads showed a downward trend in both groups, and were significantly different between the groups at the second and third detections. Additionally, the incidences of adverse events and severe adverse events were very low and not significantly different between the two groups.

Conclusion: High-dose vitamin D (1200 IU) is suitable for the prevention of seasonal influenza as evidenced by rapid relief from symptoms, rapid decrease in viral loads, and disease recovery. In addition, high-dose vitamin D is probably safe for infants.

Keywords: seasonal influenza; vitamin D; 25-hydroxyvitamin D; prevention; infant
1. Introduction

Seasonal influenza is a common respiratory tract infection caused by human influenza viruses. It affects all age groups and is a serious global public health problem, resulting in severe illness and death especially in high-risk populations [1,2]. Although infants are at a high risk of influenza-associated complications, antiviral medications are not currently approved for this age group [3,4]. Some clinical studies that have supported the use of vitamin D to prevent influenza in children have reported that nutritional supplements including vitamin D are being used for influenza prevention although the frequency and extent of their use remain unknown [5,6,7,8]. In this study, a randomized, open, controlled clinical trial was performed in order to evaluate the clinical efficacy and safety of high-dose vitamin D for the prevention of seasonal influenza A infections in infants.

2. Methods

2.1 Trial design and ethics statement

This study was a multicenter, randomized, open, controlled clinical trial, which was approved by the Institutional Ethics Committee of The First People's Hospital of Yongkang (approval number: 1-2015-11). Furthermore, the study was registered at and approved by the Registration Centre of Clinical Trials in China (approval number: ChiCTR-IOR-16009102).

2.2 Participants

We enrolled 400 infants aged 3–12 months who presented at the pediatric departments of The First People's Hospital of Yongkang, Wenzhou Medical University Affiliated Second Hospital, and Jinhua People's Hospital for healthcare follow-ups between October 2015 and May 2016. The study inclusion criteria were as follows: 1) no influenza or other respiratory tract infections within one month preceding the enrollment; 2) normal functioning of heart, liver, and
kidneys; and 3) normal baseline serum calcium and inorganic phosphorus levels. The study exclusion criteria were as follows: 1) a history of vitamin D poisoning symptoms; 2) coexisting serious diseases, including cardiac, respiratory, liver, and renal dysfunction, or severe malnutrition; and 3) the researchers determined the participant to be unsuitable for inclusion in the trial. Furthermore, we considered the following criteria for eliminating cases that were initially enrolled in the trial: 1) no longer meeting the inclusion criteria at any time point during the trial course; 2) exhibited poor compliance and did not adhere to the treatment regimen; 3) ineligible because of medical reasons or the parent’s/guardian’s decision to discontinue the trial; and 4) discontinued follow-up owing to severe adverse reactions. Infants included in the study were randomly assigned to low-dose vitamin D3 (400 IU/day) or high-dose vitamin D3 (1200 IU/day) groups, with 200 infants in each group receiving Vitamin D3 drops orally for 4 months. One drop contained 400 IU vitamin D3.

2.3 Interventions

All infants were followed-up in the pediatric health department. Infants in the low-dose and high-dose vitamin D groups received 400 IU and 1200 IU vitamin D daily for 4 months respectively. Intake of vitamin D was calculated based on the amount of vitamin D in the oral preparations, and did not include vitamin D intake from food (including milk powder) or that obtained from sun exposure. Infants in the trial did not receive any other medication to prevent influenza. At the trial initiation and subsequently, after every 2 months, serum calcium, inorganic phosphorus, and 25-hydroxyvitamin D levels were measured at the department of clinical laboratory of the hospital. Suspected influenza-like cases [9] were immediately followed up in the pediatric infectious department and the presence of respiratory viral antigens was assessed in pharyngeal secretions using an enzyme-linked immunosorbent assay (ELISA). If the infection
was determined preliminarily to be caused by the influenza A virus by ELISA, the clinical manifestations of influenza were assessed and a polymerase chain reaction (PCR) was performed to detect the virus in throat secretions. In addition, bacterial cultures of pharyngeal secretions were performed. Infants with influenza A infection received basic treatment for symptoms and vitamins C and B were also given. However, no antiviral medication, such as ribavirin or oseltamivir, was used. In cases of bacterial infection, antibiotics were offered. In cases where wheezing or bacterial infection worsened, patients were hospitalized for further treatment.

A number of parameters and symptoms were monitored as indicators of drug efficacy. Caregivers and medical staff in the pediatric infectious department initially measured the body temperature of infants once every 4 hours, but following its return to normal for 24 hours, body temperature was measured once every 8 hours. Coughing and wheezing were assessed by medical staff daily to monitor changes; routine blood examinations for white blood cell count were performed, and C-reactive protein levels were monitored. Furthermore, pathogenic detection was conducted on days 1, 4, and 7 of treatment in pharyngeal secretions using fluorescent quantitative reverse transcription-polymerase chain reaction (RT-PCR) to detect influenza viral loads. Briefly, total RNA was extracted from pharyngeal secretion samples and the M gene was amplified using the following influenza A gene-specific primers: M-1/forward (F), 5'-CGACTGCAGCGTAGCGCTT-3' and M-2/reverse (R), 5'-CATCCTGTATATGAGGCCCAT-3'. The expected amplified fragment length was 372 bp [10]. GAPDH (452 bp) served as the reference gene, and was amplified using the following primers: upstream primer/F, 5'-CCATCACCATCTTCCAGGAG-3' and downstream primer/R, 5'-CCTGCTCACCACCTTCTTG-3'.
Safety was evaluated using the following indicators: (1) Possible poisoning symptoms, including anorexia, vomiting, diarrhea, and weight loss [11], which were monitored by caregivers daily and medical staff during follow-up sessions; (2) serum calcium, inorganic phosphorus, and 25-hydroxyvitamin D levels, which were analyzed immediately following observation of poisoning symptoms; and (3) heart, liver, and kidney function as determined from biochemical blood examinations. A flow diagram of the trial design is shown in Figure 1.

2.4 Statistical analysis

Data processing and statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 17.0 software package (SPSS Inc., Chicago, IL, USA). The results of each group were compared using the Student’s t-test or Chi-square test.

3. Results

3.1 Patient demographics

We analyzed 168 and 164 infants in the low-dose vitamin D and high-dose vitamin D groups respectively, of 52.3% were male. The mean age of the infants was 7.8 ± 2.6 months. Five infants were premature, but all had a gestational age at birth of ≥32 weeks. No infants were post-term. The groups were comparable, with no significant differences in terms of sex, age, feeding patterns, location of residence, or contact with influenza-infected individuals (all P>0.05; Table 1). The treatment for 8 infants in the low-dose vitamin D group and 7 infants in the high-dose vitamin D group was interrupted because of secondary bacterial infection and hospitalization; however, all these infants were treated and no deaths occurred.

3.2 Clinical characteristics

During the 4-month of follow-up of all participants, there were 157 cases of influenza-like symptoms, including fever, coughing, a runny nose, and wheezing. All influenza-like cases were
further analyzed using an ELISA and PCR. ELISA results revealed 125 cases of influenza virus A infection and PCR revealed 121 cases; the coincidence rate of the two test results was 97.1%. In this trial, PCR results were considered the most accurate method for establishing influenza virus A infection. There were 78 and 43 cases (121 cases in total) in the low-dose vitamin D and high-dose vitamin D groups respectively, and there was a significant difference between the two groups ($\chi^2 = 14.6324, P < 0.05$). Fourteen infants with influenza A virus infections (11 in the low-dose vitamin D group and 3 in the high-dose vitamin D group) had secondary bacterial infections that progressed to pneumonia and required further treatment.

The symptoms of the influenza cases in each group, including the duration of fever, coughing, and wheezing, were compared. At trial completion, the median duration of a fever in the high-dose vitamin D group (95% confidence interval [CI], 22.18−36.77) was shorter than that in the low-dose vitamin D group (95% CI, 35.20−47.53), and the difference was significant ($t = 2.4688, P = 0.0161$; Figure 2). The median duration of coughing in the high-dose vitamin D group (95% CI, 1.42−2.57) was also significantly shorter than that of the low-dose vitamin D group (95% CI, 3.43−4.38; $t = 4.8118, P = 0.0000$; Figure 3). The median duration of wheezing in the high-dose and low-dose vitamin D groups was 1.38 ± 1.02 days (95% CI, 0.99−1.82) and 2.36 ± 1.40 days (95% CI, 2.83−3.69) respectively, and there was a significant difference between the groups ($t = 3.1142, P = 0.0018$; Figure 4).

3.3 Viral load detection of influenza virus A

Influenza virus A nucleic acids were detected using RT-PCR in throat swabs from infants with influenza-like symptoms on days 1, 4, and 7 of follow-up in both groups; the mean viral loads were compared. No significant difference was observed between the mean throat swab viral loads of the two groups for the first RT-PCR detection (6.81 ± 3.45 and 6.47 ± 3.92
respectively; $t = 1.1251$, $P = 0.2610$). Conversely, there were significant differences between the throat swab viral loads of the high-dose vitamin D and low-dose vitamin D groups at the second (1.26 ± 0.52 and 4.48 ± 1.37, respectively; $t = 40.8935$, $P = 0.0000$) and third (0.15 ± 0.04 and 1.25 ± 0.43 respectively; $t = 45.4385$, $P = 0.0000$) detections (Figure 5).

### 3.4 Detection of serum calcium, inorganic phosphorus, and 25-hydroxyvitamin D levels

Serum calcium, inorganic phosphorus, and 25-hydroxyvitamin D levels were measured three times for all infants: on the first day (first detection), during the second month (second detection), and at the end of follow-up (third detection). In China, the normal levels of serum calcium, phosphorus, and 25-hydroxyvitamin D levels are 2.25–2.77 mmol/L, 1.29–1.94 mmol/L, and 50–75 mmol/L respectively [12]. The serum calcium levels in the high-dose vitamin D group were 2.49 ± 0.18 mmol/L, 2.55 ± 0.19 mmol/L, and 2.54 ± 0.20 mmol/L in the first, second, and third detections respectively, while in the low-dose vitamin D group, they were 2.47 ± 0.19 mmol/L, 2.53 ± 0.20 mmol/L, and 2.51 ± 0.19 mmol/L respectively. There were no significant differences between the groups at each time point (first detection: $t = 1.0154$, $P = 0.3106$; second detection: $t = 0.9634$, $P = 0.3360$; third detection measured three times in the high-dose vitamin D group (1.77 ± 0.16 mmol/L, 1.73 ± 0.17 mmol/L, and 1.75 ± 0.16 mmol/L in the first, second, and third detections respectively) and in the low-dose vitamin D group (1.74 ± 0.17 mmol/L, 1.75 ± 0.18 mmol/L, and 1.72 ± 0.19 mmol/L in the first, second, and third detections respectively). There were no significant differences between the groups at each time point (first detection: $t = 1.7076$, $P = 0.0886$; second detection: $t = 1.0734$, $P = 0.2838$; third detection: $t = 1.6033$, $P = 0.1089$).

The baseline levels of 25-hydroxyvitamin D were 42.6 ± 5.9 nmol/L and 43.4 ± 6.1 mmol/L in the high-dose vitamin D and low-dose vitamin D groups respectively; $p = 0.2112$. In the second and third detections, the levels of 25-hydroxyvitamin D were higher in the high-dose
vitamin D group (61.3 ± 9.7 nmol/L and 62.8 ± 10.2 nmol/L respectively) than in the low-dose vitamin D group (43.8 ± 6.0 nmol/L and 43.1 ± 6.4 nmol/L respectively), and the differences were significant (second detection: $t = 20.3444$, p < 0.05; third detection: $t = 21.0941$, P < 0.05; Figure 6)

3.3 Safety

Four infants (two in each group) showed possible symptoms of poisoning, including vomiting and/or diarrhea. Biochemical blood examinations showed that alanine aminotransferase (ALT) levels were elevated in two of the infants. Serum calcium, inorganic phosphorus, and 25-hydroxyvitamin D levels, which were examined upon presentation of symptoms, were within the normal ranges (serum calcium level: 2.20–2.80 mmol/L; serum inorganic phosphorus level: 1.29–1.94 mmol/L; 25-hydroxyvitamin D level: 50.0–75.0 nmol/L). However, the clinical manifestations were deemed to be related to the gastrointestinal infection and not vitamin D. None of the patients exhibited circulatory, nervous, kidney, or hematologic disorders during this trial.

4. Discussion

Influenza is a viral infection that affects the general population, especially the very old and the very young [1]. In children, influenza primarily occurs in those aged <5 years [3,13]. In China, according to nationally reported data, children aged 0–5 years are frequently affected; there are 352 cases per 100,000 inhabitants [14], which is similar to reports from other countries, including the USA [15] and some countries in Europe [16]. However, no data on infant influenza have been reported [17].

A variety of prevention and antiviral treatments are currently available. The two general annual influenza vaccines available for children, including the inactivated influenza vaccine and
live attenuated influenza vaccine, are US Food and Drug Administration-approved, for children aged 1 year and older, but not for infants [18]. Adjuvant vaccines for children aged <2 years are under investigation. These vaccines aim to boost the host’s immune response to the vaccine antigen, but their efficacy is yet to be confirmed [19]. Oseltamivir is currently recommended for prophylaxis and treatment of confirmed or suspected cases of influenza among high-risk groups, including children aged <2 years [20]; however, only sparse data exists with regard to infants, and the appropriate dose ranges from 1–3 mg/kg [21,22]. Further research is required to determine the appropriate dose of oseltamivir, and to determine its safety in infants. A systematic review and meta-analysis concluded that Vitamin D supplementation was safe and protective against acute respiratory tract infections; however, this study did not analyze pathogens or focus on influenza A. Three studies were conducted on influenza A; however, they did not include infants [23].

Vitamin D has received attention because influenza is more common in winter months when diminished sun exposure results in low levels of vitamin D, although the mechanism underlying influenza seasonality has not been clearly established [24]. Vitamin D has several immunomodulatory functions, including upregulation of antiviral peptides that are part of human innate immunity and can inactivate the influenza virus [5,7]. There is a paucity of well-designed clinical studies supporting the use of Vitamin D3 to prevent influenza in children. However, no previous studies focused on infants; the participants were aged from 6 months to 5 years (600 IU of vitamin D daily for 6 months) or 6 to 15 years (1200 IU of vitamin D daily for 4 months) [5,7]. Furthermore, the previous studies had limitations, including no assessment of the baseline vitamin D levels, and the vitamin D doses in one trial may have been too low to have benefits [25]. Despite the lack of evidence, a few reports have suggested that vitamin D is currently being
used for influenza prevention; the frequency and extent of its use is unknown [26,27]. In a previous study, infants in China received 400 IU Vitamin D3 daily to prevent rickets [12]. This concentration perhaps cannot have a preventive effect against influenza, although there is no valid data. The present study evaluated the use of Vitamin D3 drops for the prevention of seasonal influenza A among infants. The participants were divided into low-dose vitamin D (400 IU) and high-dose vitamin D (1200 IU) groups.

During the course of the trial, 78 of 168 (46.4%) infants in the low-dose vitamin D group and 43 of 164 (26.2%) infants in the high-dose vitamin D group presented with influenza A infections, $\chi^2 = 14.6324$, $P = 0.0001$. Numerous factors may have led to the different infection rates, including sex; age; social distancing measures [28,29]; types of physical barrier protection (including hand hygiene, environmental cleaning, and face masks) [30,31]; and serum calcium, inorganic phosphorus, and 25-hydroxyvitamin D levels. However, we were unable to accurately evaluate social distancing measures and types of physical barrier protections, although feeding patterns, location of residence, and possible contact with influenza patients were evaluated. The majority of indicators, with the exception of 25-hydroxyvitamin D levels, were significantly different between the two groups, which might explain why the incidence of influenza A was significantly lower in the high-dose vitamin D group than in the low-dose vitamin D group, although the mechanism is anagogic and was not analyzed in the trial.

The parameters evaluated in the infants with influenza A infections included the duration of fever, coughing and wheezing, as well as viral loads of influenza A. When monitoring the reduction in the number of days with fever, the high-dose vitamin D group exhibited a faster reduction in temperature compared to the low-dose vitamin D group. Furthermore, the high-dose vitamin D group showed a more rapid improvement in the time required to resolve cough and
wheezing compared to the low-dose vitamin D group. Notably, the viral loads of influenza A in infants in the high-dose vitamin D group decreased more rapidly compared to those of infants in the low-dose vitamin D. These results suggested that high-dose vitamin D treatment exerted an antiviral effect to promote recovery.

The incidences of adverse events and severe adverse events were not statistically different between the two groups. Only four infants presented with potential poisoning symptoms, including vomiting and/or diarrhea. Biochemical blood examinations showed that ALT levels were elevated in two infants; this was considered to be due to the gastrointestinal infection and not vitamin D, thus indicating that high doses of vitamin D (1200 IU) are safe in infants. Following the end of the trial, infants were followed-up for an additional 2 months, and no sequelae were observed in the patients.
References


Figure Legends

**Figure 1. Trial design flow diagram.** AEs, adverse events.

**Figure 2. Number of patients with fever in both groups.** The total number of patients that presented with a fever in both groups was 121, including 78 in the low-dose vitamin D group and 43 in the high-dose vitamin D group. The interval between each time point was 4 hours. Temperatures >37.5°C were considered to indicate a fever and those maintained at <37.5°C for >24 hours were considered normal.

**Figure 3. Number of patients with cough in both groups.** The total number of patients that presented with a cough in both groups was 109, including 70 in the low-dose vitamin D group and 39 in the high-dose vitamin D group. The disappearance of a cough was considered a return to normal.

**Figure 4. Number of patients with wheezing in both groups.** The total number of patients that presented with wheezing in both groups was 93, including 62 in the low-dose vitamin D group and 31 in the high-dose vitamin D group. The disappearance of wheezing was considered a return to normal.

**Figure 5. Influenza A viral loads in throat swab samples from both groups.** The total number of patients that presented with a fever in both groups was 121, including 78 in the low-dose vitamin D group and 43 in the high-dose vitamin D group. *P<0.05 vs. the high-dose vitamin D group. The first, second, and third detections involved analysing throat swab samples in triplicate using reverse transcription-polymerase chain reaction on days 1, 4, and 7 of follow-up.
Figure 6. 25-hydroxyvitamin D levels in both groups. The levels of 25-hydroxyvitamin D were significantly higher in the high-dose vitamin D group (n=164) than in the low-dose vitamin D group (n=168) in the second and third detections. *P<0.05 vs. the low-dose vitamin D group.
<table>
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<th>Variable</th>
<th>Low-dose vitamin D (n=168)</th>
<th>High-dose vitamin D (n=164)</th>
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<td>79 (47.0)</td>
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<td><strong>Age (months)</strong></td>
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</table>
Figure 1

Enrolled and randomised (n = 400)

Allocated to low-dose vitamin D group (n = 200)
- Discontinued intervention (n = 17)
- Interruption of treatment (n = 8)
- Poor compliance (n = 5)
  - Analysed (n = 168)

Allocated to high-dose vitamin D group (n = 200)
- Discontinued intervention (n = 20)
- Interruption of treatment (n = 7)
- Poor compliance (n = 7)
  - Analysed (n = 164)
Figure 2

- **low dose Vitamin D group**
- **high dose Vitamin D group**

- Number of patients with fever
- Number of hours since onset of illness

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Figure 3

The graph shows the number of patients with coughing over the days of illness.

- Solid black line: low dose Vitamin D cases
- Red dotted line: high dose Vitamin D cases
Figure 4

- Low dose Vitamin D group
- High dose Vitamin D group

Number of patients with wheezing vs Day of illness
Figure 5

The viral load of influenza A ($\times 10^6$ copies/ml)

- low dose Vitamin D group
- high dose Vitamin D group

Time of viral assessment

1.
Figure 6

![Graph showing 25-hydroxyvitamin D levels over time in low dose and high dose Vitamin D groups.](image)

- **y-axis**: 25-hydroxyvitamin D level (nmol/L)
- **x-axis**: Time of 25-hydroxyvitamin D assessment

Legend:
- Black square: low dose Vitamin D group
- Red circle: high dose Vitamin D group

Note: The graph indicates a significant increase in 25-hydroxyvitamin D levels in the high dose group compared to the low dose group at time 2 and time 3, marked with an asterisk.