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Original Article

A randomized trial of vitamin D supplementation to prevent seasonal influenza and enterovirus infection in children

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KEYWORDS
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Abstract *Purpose:* This study aimed to evaluate whether vitamin D supplementation can reduce the incidence of influenza and enterovirus infection in Taiwanese children.
Methods: This randomized, double-blind, controlled trial included children aged two to five years between April 2018 and October 2019 from daycare centers. All the participants were randomly assigned to a vitamin D supplementation group (2000 IU/day) or placebo group for one month. The primary outcome was the incidence of influenza and enterovirus infection in the following six months, and the secondary outcome was the incidence of influenza and enterovirus infection in the children's household members.
Results: Two hundred and forty-eight children participated. The vitamin D group showed a relative risk reduction of 84% against influenza compared to the placebo group but did not reach statistical significance. Kaplan–Meier curves revealed that the placebo group had a higher probability of influenza infection than the vitamin D group (log-rank test, $p = 0.055$), but the incidence of enterovirus infection was similar between the two groups ($p = 0.946$) among children. Among children's household members, the incidence of influenza ($p = 0.586$) and enterovirus infection ($p = 0.528$) were both similar between the two groups. All children

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who were tested for serum 25(OH)D levels after vitamin D intervention had 25(OH)D levels above 30 ng/ml
Conclusion: Vitamin D supplementation may have a small preventative effect against influenza infection but does not affect enterovirus infection among preschool children. A high-dose short-term vitamin D intervention might be a way to elevate children's serum vitamin D levels in the first month of starting kindergarten.

Introduction

Influenza and enterovirus infection have been two major health issues in children under five years of age in Taiwan. Young children suffer considerable morbidity from influenza, with potential consequences for their siblings, parents, and other caregivers in terms of secondary infections and caregiver leave.[1–3] The case fatality rate of severe enterovirus disease ranged from 11% to 33.3% from 1998 to 2018, mostly due to enterovirus 71, the most common serotype causing severe disease.[4,5] In Taiwan, a 5 to 7-day proactive kindergarten closure will be executed if more than two children a class are diagnosed with enterovirus or influenza infection. The endemic control policy causes a lot of chaos in both the teachers' and parents' lives. Thus, how to prevent influenza and enterovirus infection among children is an important issue in Taiwan.

Recent studies have demonstrated that vitamin D plays an important role in innate and adaptive immune responses to fight against infections. Vitamin D may interfere with viral infections by interacting with cell entry receptors. The effects of vitamin D on reducing inflammation and strengthening the immune system have been implied in both respiratory tract infections and during the COVID-19 pandemic.[6,7] Moreover, vitamin D deficiency has been associated with an increased risk of acute respiratory tract infections both in adults[8] and children.[9]

The prevalence of vitamin D insufficiency in Taiwanese children is high throughout the first three years of life (60% at one year to 44% at three years of age)[10] and in school-age children (five to eighteen years of age, 51%).[11] High-dose vitamin D supplements have been reported to be effective in the prevention of seasonal influenza in infants aged three to twelve months[12] and children aged six to fifteen years.[13] In addition to preventing influenza infection, vitamin D supplements have also been reported to play an important role in reducing other viral infections such as entero-rhinovirus infections.[14]

Few well-designed clinical studies have investigated the use of vitamin D supplements to prevent influenza and enterovirus infections in preschool-aged children, who are a major risk group when infected. Herein, we conducted a randomized controlled trial to evaluate whether vitamin D supplementation can reduce the incidence of influenza and enterovirus infection in Taiwanese children from two to five years of age.

Methods

We conducted this trial to compare the effects of vitamin D supplementation on influenza and enterovirus diseases with a placebo group given purified coconut oil. The initial study design was to compare the class closure days due to enterovirus or influenza outbreaks during the study period. We used random permuted blocks to randomly assign classes into intervention or control groups. However, unexpectedly, most of the parents refused to join the study, even with the extremely low invasiveness, so that less than half of the children in each class were included. Therefore, we skipped the primary endpoint and moved on to analyze the disease incidence instead.

From April 2018 to October 2019, those preschool-age children from one of the biggest kindergarten chains in northern, central, and southern Taiwan were invited to join in this prospective study. The participants were randomly assigned into two groups: a vitamin D supplementation group and a placebo group.

Identical bottles with 60,000 IU of vitamin D or 5 ml of purified coconut oil were prepared. The vitamin D was dissolved in coconut oil formulation so that there was no difference in taste between the contents. In this double-blind study, neither the teachers nor the parents knew the contents of the bottles. The parents were given instructions in a leaflet and an online video on how to give their children drops of the vitamin D or purified coconut oil. The intervention group took 2000 IU of the vitamin D supplementation per day for one month, and the placebo group took the purified coconut oil for one month. We also asked the parents to complete a questionnaire on their child, including age, gender, height, weight, history of illness, exposure time, number of siblings, lifestyle, etc. On the 15th and 30th days after the starting date, the researchers contacted the parents and checked on the remaining amount of vitamin D or coconut oil left in the bottles to ensure that the children were taking them on schedule. After finishing the bottles, we invited families in both groups to bring their children and test their serum 25(OH)D concentration. The serum 25(OH)D levels were tested at the MacKay Memorial Children's Hospital laboratory.

The research team contacted the teachers and parents every month and asked them to report any influenza or enterovirus infections during the six months follow-up period. During the telephone interview, we confirmed with

the parents whether their child had received a physician-administered influenza rapid test or a physician-certified diagnosis of influenza or enterovirus. Only lab-confirmed influenza illnesses were recorded, while enterovirus infections could be clinically diagnosed by a family doctor. We also record secondary infections among household members and recorded their symptoms. The outcome of interest was the incidence of influenza and enterovirus diseases in the following three (90 days) and six months (180 days).

Statistical analysis was conducted using IBM SPSS software version 25 (IBM Corp., Taipei, Taiwan). Descriptive statistics included number, percentage, median, range, mean, and standard deviation (SD). Categorical variables were analyzed using the chi-square test and Fisher's exact test, and continuous variables were analyzed using the student's t-test. Kaplan–Meier analysis (log-rank test) was used to compare the probability of the incidence of influenza or enterovirus infection, respectively, during the six-month observation period in both groups (vitamin D/placebo). The probabilities of the incidence of disease in household members were also analyzed. A Cox regression model was used to adjust for relevant covariates between the two groups. The cases without either influenza or enterovirus infection in six months were regarded as right-censored data. The analysis used an intention to treat population; all missing data were regarded as being an event that did not occur (right-censored data). Comparisons with a p-value < 0.05 were considered to be statistically significant. This study was approved by the Institutional Review Board of MacKay Memorial Children's Hospital (number: 17MMHIS179e).

Results

Characteristics of the study population

There were no significant differences in sex, number of people in the family, number of siblings, age of siblings, gestational age, education status of the mother, siblings in the same kindergarten, outdoor hours per week, nutritional supplementation, snack eating habits, dairy product consumption, influenza vaccination status and underlying diseases between the two groups. The age of the children in the vitamin D group (4.03 $\pm$ 0.67 years) was significantly older than that in the placebo group (3.76 $\pm$ 0.75 years) ($p = 0.003$) (Table 1). Of all enrolled participants, 198 (79.8%) completed six months of follow-up. Numbers of children recruited by month are shown in Fig. 1. The study flow diagram is shown in Fig. 2. A total of 248 healthy children who met the inclusion criteria agreed to join this study, including 225 from northern and 23 from southern Taiwan. Of the 50 participants who withdrew from the study, 25 were in the vitamin D group and 25 were in the placebo group (Fig. 2). None of these withdrawals were due to adverse events, and no adverse effects were noted in any of the children during the study.

Incidence of disease in the six months of follow-up

The vitamin D group showed a relative risk reduction (RRR) of 84% (95% CI: −0.412, 0.980) against influenza compared with the placebo group among the study cases, and a RRR of 16.5% against influenza among the household members (95% CI: −0.858, 0.623). The incidence of influenza infection among the cases was lower in the vitamin D group, but the difference did not reach statistical significance (vitamin D group 1/135 (0.7%), placebo group 5/113 (4.4%); $p = 0.095$, Table 1). No age difference was found in the children with (n = 6) and without (n = 242) influenza infection (influenza: 3.98 $\pm$ 0.72 years, non-influenza: 3.90 $\pm$ 0.72 years; $p = 0.795$). The incidence rates of influenza infection among the household members in the two groups were similar (vitamin D group 11/135 (8.1%), placebo group 11/113 (9.7%); $p = 0.662$, Table 1).

On the other hand, the incidence rates of enterovirus infection were similar between the two groups (vitamin D group 17/135 (12.6%), placebo group 14/113 (12.4%); $p = 0.962$), and among the household members (vitamin D group 7/135 (5.2%), placebo group 8/113 (7.1%); $p = 0.533$, Table 1).

Kaplan–Meier analysis

During the six-month follow-up period, Kaplan–Meier curves revealed that the placebo group had a higher probability of influenza infection than the vitamin D group, but the difference was not significant (log-rank test, $p = 0.055$, Fig. 3A). Among the household members, the Kaplan–Meier curves revealed no significant difference in influenza between the two groups (log-rank test, $p = 0.586$, Fig. 3B). Kaplan–Meier curves also revealed no significant difference regarding enterovirus infection in the children (log-rank test, $p = 0.946$, Fig. 3C) or household members (log-rank test, $p = 0.528$, Fig. 3D).

Cox regression model

We used a Cox regression model to adjust for the age difference between the vitamin D and placebo groups. The results showed that younger age was not a risk factor for influenza infection ($p = 0.538$, Supplement Table 1).

Vitamin D level

We planned to draw the baseline blood sample for the serum 25-(OH)D and plasma calcium level. However, we faced resistance from the parents. Therefore, we tried to invite families to voluntarily bring their children and test their serum 25(OH)D concentration after the intervention, and a total of 21 children volunteered (nine in the intervention group and 12 in the placebo group). We only checked Vit D serum levels once at one month after completing the intervention. Of the 21 children who were tested for serum 25(OH)D levels, all of those in the vitamin D intervention group (9/9, 100%) had 25(OH)D levels >30 ng/mL, while 67% (8/12) of those in the placebo group had a 25(OH)D level of 10–20 ng/ml, and 33% (4/12) in the placebo group had 25(OH)D levels >20 ng/ml (Table 2).

Discussion

In this study, we found that daily supplementation with 2000 IU vitamin D for one month may have offered a

Table 1 Demographic characteristics and incidence of Influenza and enterovirus.

Variables	Placebo group	Vitamin D group	*p*-value
	n = 113 No. (%)	n = 135 No. (%)	
Age (mean ± SD) (years)	3.76 ± 0.75	4.03 ± 0.67	[a]0.003
Gender			
Male	69(61.0)	67(49.6)	0.072
Female	44(38.9)	68(50.4)	
Number of family members			
≤ 3	28(24.8)	32(23.7)	0.279
4	39(34.5)	61(45.2)	
5	18(15.9)	18(13.3)	
≥ 6	28(24.8)	23(17)	
NA	0(0.0)	1(0.7)	
Number of siblings			
0	29(25.7)	36(26.7)	0.588
1	66(58.4)	78(57.8)	
2	13(11.5)	12(8.9)	
≥3	3(2.7)	8(5.9)	
NA	2(1.8)	1(0.7)	
Siblings' age (years)			
None	28(24.8)	36(26.7)	
< 2	19(16.8)	31(23.0)	
2—5	30(26.5)	24(17.8)	
5—10	28(24.8)	33(24.4)	0.483
≥ 10	7(6.2)	9(6.7)	
NA	1(0.9)	2(1.5)	
Term baby			
No	8(7.1)	9(6.7)	0.936
Yes	105(92.9)	123(91.1)	
NA	0(0.0)	3(2.2)	
Congenital anomaly or chronic disease			
No	103(91.2)	123(91.1)	0.704
Yes	10(8.8)	10(7.4)	
NA	0(0.0)	2(1.5)	
Mother's education level			
Masters and above	8(7.1)	18(13.3)	0.092
University	72(63.7)	93(68.9)	
College	18(16.0)	11(8.1)	
High school	15(13.3)	13(9.6)	
Junior high school and below	0(0.0)	0(0.0)	
NA	0(0.0)	0(0.0)	
Siblings at the same kindergarten			
No	80(70.8)	106(78.5)	0.162
Yes	33(29.2)	29(21.5)	
NA	0(0.0)	0(0.0)	
Mean time of sun exposure (hours/week)			
< 7	40(35.4)	64(47.4)	0.262
7—14	56(49.6)	52(28.5)	
14—21	12(10.6)	17(12.6)	
21—28	2(1.8)	2(1.5)	
≥ 28	1(0.9)	0(0.0)	
NA	2(1.8)	0(0.0)	
Average sun exposure time			
before 10:00	29(25.7)	39(28.9)	0.511
10:00—14:00	34(30.1)	38(28.1)	
after 14:00	49(42.4)	57(42.2)	
NA	1(0.9)	1(0.7)	

Table 1 (*continued*)

Variables	Placebo group	Vitamin D group	*p*-value
	n = 113 No. (%)	n = 135 No. (%)	
Sun exposure time on holiday			
before 10:00	24(21.2)	32(23.7)	0.887
10:00–14:00	36(31.8)	39(28.9)	
after 14:00	51(45.1)	62(45.9)	
NA	2(1.8)	2(1.5)	
Supplements			
None	36(31.9)	40(29.6)	0.560
Probiotic	64(56.6)	83(61.5)	
Complex vitamins	0(0.0)	1(0.7)	
Vitamin C	7(6.2)	3(2.2)	
Vitamin D	0(0.0)	0(0.0)	
Calcium tablets	2(1.8)	2(1.5)	
Enzyme	0(0.0)	0(0.0)	
Fish oil	1(0.9)	1(0.7)	
Cod liver oil	1(0.9)	1(0.7)	
Propolis	1(0.9)	0(0.0)	
Others	0(0.0)	4(3.0)	
NA	1(0.9)	0(0.0)	
Snack habits			
None	3(2.7)	5(3.7)	0.223
Every day	42(37.2)	42(31.1)	
3–5 times/week	46(40.7)	52(38.5)	
1–2 times/week	21(18.6)	31(23.0)	
< 2 times/month	0(0.0)	5(3.7)	
NA	1(0.9)	0(0.0)	
Drinking dairy products			
No	14(12.4)	21(15.6)	0.74
Whole fat milk	40(35.4)	42(31.1)	
Low fat milk	0(0.0)	1(0.7)	
Milk formula	41(36.3)	50(37.0)	
Breast milk	1(0.8)	1(0.7)	
Others	14(12.4)	17(12.6)	
NA	3(2.7)	3(2.2)	
Receiving seasonal influenza vaccination			
Trivalent vaccine	46(40.7)	48(35.6)	0.605
Quadrivalent vaccine	10(8.8)	15(11.1)	
No vaccine	54(47.8)	71(52.6)	
NA	3(2.7)	1(0.7)	
Children had influenza infection during the 6-month follow-up period			
No	108(95.6)	134(99.3)	0.095
Yes	5(4.4)	1(0.7)	
Household members had influenza infection during the 6-month follow-up period			
No	102(90.3)	124(91.9)	0.662
Yes	11(9.7)	11(8.1)	
Children had enterovirus infection during the 6-month follow-up period			
No	99(87.6)	118(87.4)	0.962
Yes	14(12.4)	17(12.6)	
Household members had enterovirus infection during the 6-month follow-up period			
No	105(92.9)	128(94.8)	0.533
Yes	8(7.1)	7(5.2)	

[a] p (chi-square).
Abbreviations.
No., number; SD, standard deviation; NA, not available.

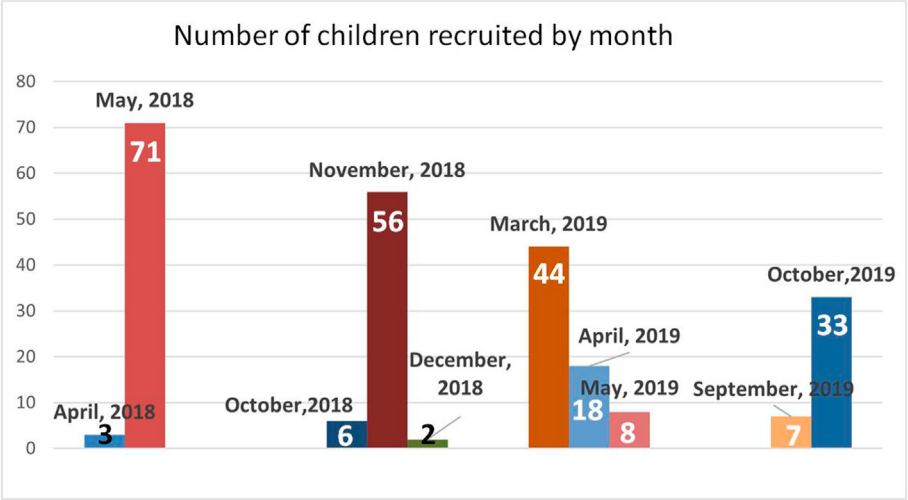

Figure 1. Number of children recruited by month.

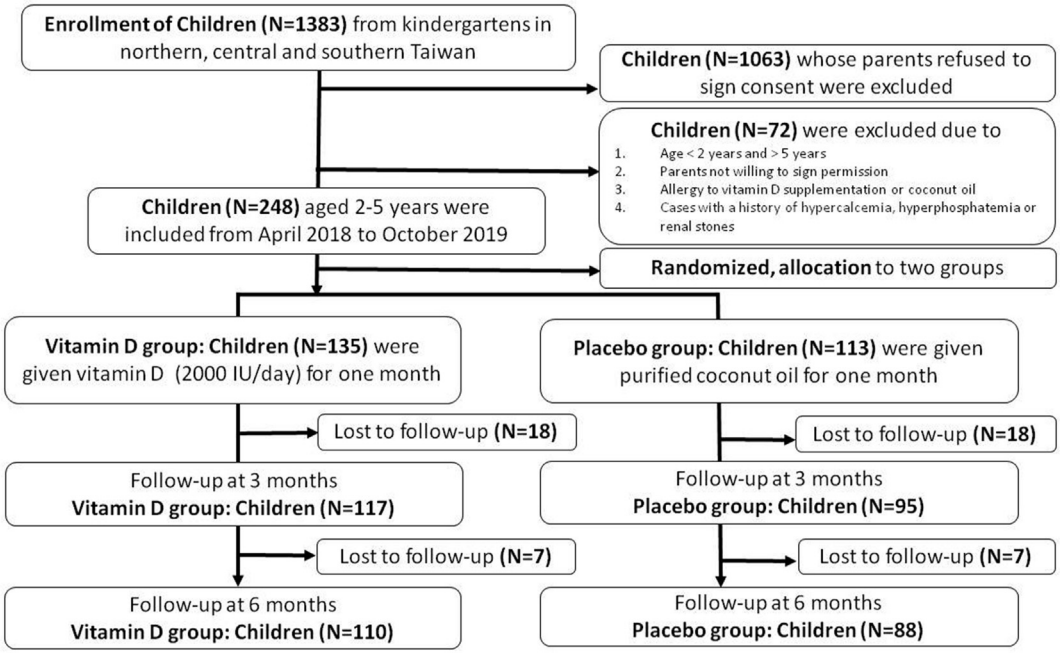

Figure 2. Study recruitment flow chart.

positive but not significant preventive effect against influenza during 180 days after the intervention. There was no significant preventive effect against enterovirus infection in our study.

In the past decade, studies have shown that various diseases are associated with vitamin D deficiency.[15,16] As with rickets, for example, vitamin D deficiency has also been associated with an increased risk of acute respiratory tract infections both in adults[8] and children.[9] Arihiro et al. reported that vitamin D supplementation may have had a preventative effect against upper respiratory infections in their cohort and that the effect was more prominent in patients whose vitamin D levels were below 20 ng/ml.[17] Moreover, children with vitamin D levels below 10 ng/ml have been reported to be 11 times more likely to be infected with lower respiratory infections than healthy children, especially those under five years of age.[9,18]

Several possible mechanisms may explain the potential preventive effect of vitamin D against influenza infection. First, vitamin D can modulate the innate and acquired immune systems by attaching to vitamin D receptors (VDRs) in various human immune cells.[6] The VDRs then induce the activation of Toll-like receptors (TLRs), which play an important role in recognizing pathogens.[18–20] Such activated TLRs release cytokines, which then induce antimicrobial peptides such as defensins and cathelicidins.[21] Defensins have an attractive effect on neutrophils and monocytes which can both inactivate the pathogenic effect of the influenza virus.[21] In addition, cathelicidins have a destructive effect on the envelope proteins of the influenza

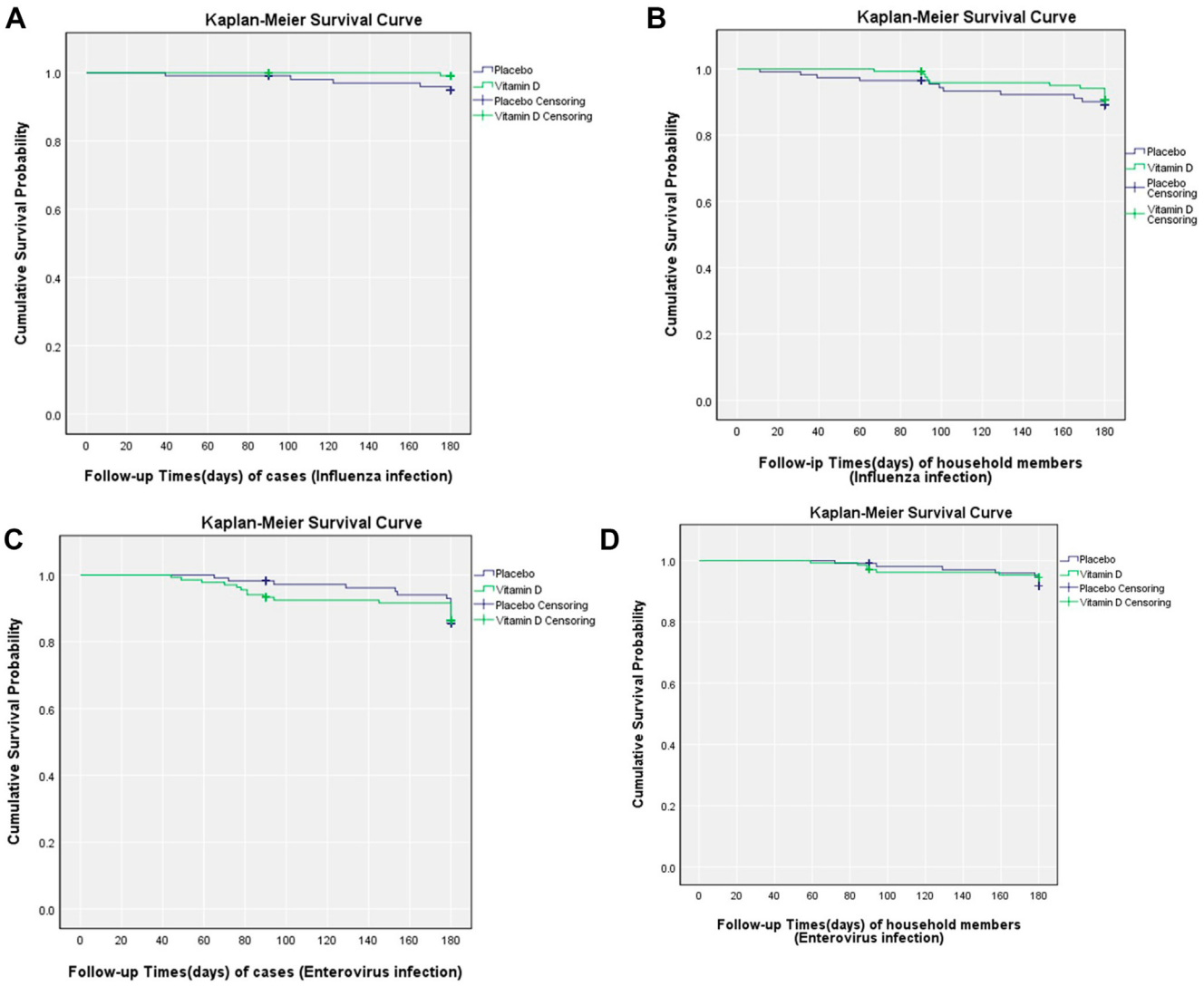

Figure 3. Kaplan–Meier analysis was used to compare probabilities of infection in the two groups (vitamin D/placebo). The Kaplan–Meier curves show the probabilities of the incidence of influenza (Fig. 3A) or enteroviral infection (Fig. 3C), respectively. Analysis of the probabilities of infection in the household members for influenza (Fig. 3B) and enterovirus (Fig. 3D). Censoring data is indicated by cross marks.

Table 2 Serum 25(OH)D serum level.

	Placebo group (n = 12)	Vitamin D group (n = 9)
	n (%)	n (%)
10–19 ng/ml	8 (67%)	0(0%)
20–29 ng/ml	2(16.5%)	0(0%)
≥ 30 ng/ml	2(16.5%)	9(100%)

Deficiency: 25(OH)D < 20 ng/ml (50 nmol/L).
Sufficient: 25(OH)D ≥ 20 ng/ml (50 nmol/L).

virus.[19] These mechanisms may support the possible protective effect of vitamin D supplementation against influenza infection among children.

One review article of several randomized controlled trials from 2010 to 2019 investigated the preventive effect of vitamin D supplementation against influenza and COVID-19 illnesses. Grant et al. reported that vitamin D supplementation could reduce the risk of influenza and/or COVID-19 in high-dose (10,000 IU/day), short-term (few weeks) interventions. High serum vitamin D levels are crucial in reducing influenza and/or COVID-19 infection.[22] Several studies from this review article on children/adolescents reported that vitamin D could reduce the incidence of influenza infection in children aged six to fifteen years[13] and infants aged three to twelve months.[12] In addition, Urashima et al. reported that a four-month vitamin D intervention with a dosage of 1200 IU/day in school-age children had a preventive effect after 15–17 weeks of follow-up, reducing the incidence of influenza by 42% among children aged six to fifteen years. The protective effect was even more prominent in children who started daycare centers after three years of age (RR: 0.36; 95% CI: 0.17, 0.78; $p = 0.005$).[13] Different from Urashima's study,

we focused on a younger population (two to five years of age). Although we also observed a RRR of 84% against influenza, the number of infected cases was too small to reach statistical significance.

The high-dose (2000 IU/day), short-term (one month) intervention we used in our trial was inspired by a study from Zhou et al., who designed a four-month, 1200 IU/day vitamin D intervention to prevent influenza infection and reported positive results in infants.[12] Other studies have also shown that a high-dose vitamin D intervention (2000 IU/day) can increase serum vitamin D levels to normal levels within one month[23,24] without safety concerns such as hypercalcemia.[25] In addition, Vogiatzi et al. reported that after a single dose of 50,000 IU vitamin D, the serum 25(OH)D concentrations could reach above 30 ng/mL after 28 days in pediatric patients below 21 years of age.[23,] Maalouf et al. also reported no significant difference in 25(OH)D concentrations between a short-term (14,000 IU/week for 8 weeks) intervention and long-term intervention (14,000 IU/week for one year) with vitamin D supplementation. The effect of a short-term intervention has been reported to be non-inferior to a long-term intervention in raising vitamin D serum levels.[25]

In our study, all (9/9, 100%) of the children who were tested for 25(OH)D in the vitamin D group had a serum 25(OH)D concentration $>$30 ng/mL in one month, while 67% of the children in the placebo group still had vitamin D deficiency.[26] Although the sample size is small, the percentage of vitamin D deficiency in the placebo group seemed to be coherent to the result from previous studies.[11] Based on the studies by Liao et al. and Yao et al., the prevalence of vitamin D insufficiency in Taiwanese children is high throughout the first three years of life (60% at one year to 44% at three years of age) [7] and in school-age children (five to eighteen years of age, 51%). [8] Since none of the 25(OH)D-tested participants in the intervention group had vitamin D deficiency, we believe that a high-dose short-term intervention is a practical way to elevate children's serum vitamin D levels in the first month of starting kindergarten or daycare.

Studies on the preventive effect of vitamin D supplementation on preventing influenza infections are not always consistent. For example, Loeb et al. reported that vitamin D did not reduce the incidence of influenza among children aged three to seventeen years in Vietnam (hazard ratio [HR]: 1.18, 95% CI: 0.79—1.77, log-rank $p = 0.64$).[14] However, the baseline vitamin D deficiency rate was as low as 17% in their study population, compared to approximately 67% in our study. The older mean age (8.5 years) in Loeb's study may also have affected the efficacy of vitamin D supplementation, since a previous infection or immunization may render a certain level of protection. We believe that our results could be helpful for pediatricians, parents, and teachers of two to five-year-old preschoolers who live in regions with high vitamin D deficiency rates, although more studies are needed to prove the concept.

Vitamin D supplementation did not have a preventive effect against enterovirus infection in our study. Even though Loeb et al. found that vitamin D supplementation significantly reduced RT-PCR-confirmed non-influenza respiratory viral infections including entero-rhinovirus (HR: 0.76, 95% CI: 0.61—0.94, log-rank $p = 011$), in this case, rhinovirus may have been the pathogen rather than enterovirus.[14] However, Federico et al. found that serum vitamin D levels are crucial in reducing enterovirus infection among diabetic children/adolescents.[27] Further randomized controlled trials are needed to elucidate this issue.

There are many limitations to this study. First, the small sample size made it difficult to reach significant power. The sample size was designed to be 350 subjects in each group, but the recruitment did not go well, and only 248 subjects participated. Second, the serum level of vitamin D was measured in volunteers, resulting in uneven distribution of vitamin D deficiency between the two groups which may have influenced the results. Third, the laboratory tests were done by the children's household doctors, not by the investigators. It is possible that some of the children with flu may have gone undetected in our study if their family doctors did not perform RIDT (Rapid Influenza Diagnostic Test), leading to underestimation of the number of flu cases in both the intervention and placebo groups. Although at the beginning of the study we suggested that the parents could ask their family doctors to perform RIDT for their children when flu-like illnesses occurred, it was impossible to ensure that each individual physician followed our suggestions. Fourth, even after double confirmation with the teachers and parents, recall bias and misdiagnosis of enteroviral infections were still possible. Fifth, since the COVID-19 pandemic occurred in January 2020, the 40 children recruited around September and October would have been affected by the pandemic during the follow-up period. Wearing face masks and improving hygiene habits for pandemic control drastically reduced the overall incidence of infectious diseases, and this may also have affected the results.

Conclusion

Vitamin D supplementation may have a small preventative effect against influenza infection but did not affect enterovirus infection among the preschool children in this study. A high-dose short-term vitamin D intervention could be a practical way to elevate children's serum vitamin D levels in the first month of starting kindergarten or daycare, although we do not have the baseline vitamin D levels.

Declaration of competing interest

The authors declare no potential conflicts of interest with regards to the research, authorship, or publication of this article. The vitamin D (trade name: Youbaodi) used in this experiment is a nutrient, imported as a food, not a medicine.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.01.003.