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Enhancement of vitamin A combined vitamin D supplementation on immune response to Bacille Calmette-Guérin vaccine revaccinated in Chinese infants

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ABSTRACT

Objective: To investigate whether there is an association between diameter of bacille Calmette-Guérin (BCG) scars and effect of purified protein derivative (PPD) reaction and to determine whether vitamin A (VA) combined vitamin D (VD) supplementation influences the immune response to BCG revaccinated in Chinese infants. **Methods:** A cross-section and 3-month community-randomised trial was conducted. A total of 5 629 infants at 3, 6 and 12 months of age in Junan County of China were examined for BCG scar formation. Then, 597 revaccinated infants were randomly assigned to supplementation (n=307) and control (n=290) groups. The supplementation group were daily assigned to 1 500 IU VA and 500 IU VD for 3 months. Then all infants were subjected to skin test with PPD. **Results:** The diameter of BCG scars was positively correlated with diameter of skin indurations of PPD (r=0.17, P<0.05) in the 5 629 infants. The rate of positive response to PPD was higher in the supplementation group than in the control group (96.1% versus 89.7%, P<0.05, prevalence ratio 1.07, 95% *CI* 1.02-1.12). The prevalence ratio of PPD response for the supplementation group compared with that for the control group was 1.07 (95% *CI* 1.01-1.13) for the males and 1.08 (95% *CI* 1.00-1.17) for the females. For the supplementation group, the males got larger tuberculin induration than the females [(0.73±0.21) cm versus (0.67±0.20) cm, P<0.05) after intervention. **Conclusions:** The diameter of BCG scars was effectively correlated with PPD response, which indicates BCG scar formation may be an useful tool to evaluate the effect of tuberculosis prevention.VA combined VD supplementation may play an immuno-regulatory role in BCG revaccination. This may contribute to the prevention of childhood tuberculosis.

1. Introduction

Bacille Calmette-Guérin (BCG) vaccination is administered in infancy in China with the aim to protect against mycobacterial infections such as tuberculosis (TB) and leprosy[1]. Failure of BCG vaccination and little effect on preventing TB are contributed to the influence of environmental mycobacteria, differences between BCG strains, inefficient immune response after BCG stimulation, genetic factors and malnutrition of populations[2,3].

Micronutrient deficiencies like vitamin A (VA) and vitamin D (VD) are more common in TB patients[4,5]. In recent years, there has been renewed interest in the biological effects of VA and VD on TB due to the growing evidence of the immunomodulatory properties of VA and

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VD. VA supplementation has been shown to modulate T helper 2 lymphocyte responses in childhood[6]. The active VD metabolite, 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$], has been shown to be an important regulator of innate and adaptive immune function and modulates the host response to *Mycobacterium tuberculosis* infection. Toll like receptor mediated activation of macrophages up-regulates expression of the VD receptor, leading to the induction of the antimicrobial peptide cathelicidin and growth restriction of *Mycobacterium tuberculosis*[7].

The correlation to vaccine efficacy may be affected by sex. For instance, boys develop scars and purified protein derivative (PPD) responses more frequently than girls[8], but there is no evidence boys are better protected against TB after BCG vaccination. Some previous studies have suggested sex-differential effect of VA and on response to vaccines, when the analyses were conducted separately for each sex[9-12].

As the induced immunity is mainly cellular, the immune response to BCG vaccination is difficult to be quantified. Instead, scar development and response to an immunogenic component of BCG, PPD of *Mycobacterium tuberculosis*, are used to measure vaccine response in epidemiologic studies. Both scar development and PPD response were reported correlated positively with a history of BCG vaccination in Asia population[13].

We aimed to investigate whether there is an association between diameter of BCG scars and effect of PPD reaction and to examine whether VA combined VD can influence the immune response to BCG revaccination evaluated as scar formation and response to PPD *in vivo* in infants.

2. Material and methods

2.1. Subject selection

Ethical permission for all aspects of this research was obtained from the Ethic Review Committees of Center for Disease Control of Junan County, and studies were conducted according to the principles outlined in the Declaration of Helsinki. Parents of study subjects were informed, and each final participant submitted informed consent before the trials. Healthy infants, who did not respond to PPD test at 3, 6, 12 months of ages respectively, were enrolled in the intervention study. Exclusion criteria included severe or chronic illness, moderate to severe injury, surgery during the previous month, chronic renal failure, liver disease, heart failure, irritability of BCG, and history of other preventive inoculation less than 2 weeks.

2.2. Enrollment

Follow the policy of government, all infants in Junan County, Shandong Province were vaccinated intradermally in the upper left deltoid region with 0.1 mL BCG vaccine (contains 5 tuberculin units) at birth. A total of 5 629 infants at 3, 6 and 12 months of age in 20 towns of Junan County from 2011 to 2012 were enrolled in our study.

2.3. Anthropometrics

Measurements were made by trained assistants when the infants were taken to local centers for disease control. The length of the infants was measured supinely using a wooden measuring board. The weight of the undressed infant was measured by weighing machine. The head circumference was measured by using a tape rule.

2.4. Scar formation and in vivo PPD response

Local assistants documented health status, health care contacts, household characteristics and vaccination status and also measured the size of the BCG scar. The BCG scars of all infants were measured. Subsequently, 0.1 mL PPD was injected intradermally on the ventral side of the left forearm. Three days later, the infant was taken to the center again, and the induration was measured by trained assistants. The infants with a PPD response ($\geqslant$0.5 cm) were categorized as "PPD-responders". Diameter of skin indurations of PPD $\geqslant$ 1.5 cm accompanied with blister or necrosis was judged as a strong response, and X-ray and sputum examination were used to determine whether the infants had been infected by *Mycobacterium tuberculosis*.

2.5. Intervention assay

A community-randomised trial was performed. Of the 5 629 infants, 608 infants did not respond to PPD test (<0.5 cm) and needed to be revaccinated. Because of the difficulty to find a legal drug manufactory to make placebo in rural area of China, the double-blind trials were not used. Randomization was applied to the entire 20 towns of Junan County (using a random number generator). The infants in 10 towns were divided into supplementation group, while those in other 10 towns into control group. One infant had moved to another place and parents of 10 infants refused to participate into intervention. Finally, 307 infants were enrolled in the supplementation group and 290 in the control group. All subjects were given BCG revaccination. The subjects in the supplementation group received VA (1 500 IU) combined VD (500 IU) drops (Qingdao Double Whale Pharmaceutical), 1

capsule/d for 3 months. The drops were distributed by staffs from local centers for disease control. The infants were taken to give PPD re-test after 3 months.

2.6. Statistical analysis

All the statistical analysis was performed using SPSS, version 11.5. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Length, weight, head circumference and other summarized data are expressed as mean ± standard deviation (SD). Diameter of tuberculin induration of different age groups was analyzed by one-way ANOVA. Size of scar and PPD was analyzed using Student's *t* tests between and within the intervention groups. Prevalence of PPD response between the intervention groups was used *Chi*-square test. Estimates were reported as prevalence ratio with 95% confidence intervals (*CI*) after intervention for the supplementation group compared with the control group. Correlations between BCG scar formation and tuberculin induration were estimated using Pearson's correlation coefficient. Differences and associations were considered significant at $P<0.05$.

3. Results

3.1. Effect of age on scar formation and PPD response

Details of scar formation and tuberculin testing at each age group are shown in Table 1. Real scar formation (>0.2 cm) were developed in 5 119 (90.9%) of the vaccinated infants, a tiny scar (≤0.2 cm) in 400 (7.1%), and no scar in 110 (2.0%). About 89.2% (5 021/5 629) infants responded to PPD (≥0.5 cm). Totally, 4 994 (88.7%) subjects gave a positive tuberculin reaction, 27 (0.5%) gave a strong positive induration, and 608 (10.8%) gave a PPD-negative response. X-ray and sputum examination revealed no evidence of active TB in 65 infants with a tuberculin induration greater than 1.5 cm or displaying blister or necrosis.

The median scar size was larger in the males than in the females [(0.40±0.15) cm versus (0.38±0.13) cm, $P<0.05$] in the 6 months group. The rate of response to PPD was higher in the males than in the females in the 3 months group (92.0% versus 88.7%, $P<0.05$) but was higher in females than in males in the 6 months group (88.1% versus 91.5%, $P<0.05$). The relation between sex and diameter of tuberculin induration showed no differences in all age groups.

3.2. Correlation between tuberculin induration and scar formation

There was a positive correlation ($r=0.17$, $P<0.05$) between scar formation and tuberculin induration (Figure 1). About 90.3% of the infants with scar formation responded to tuberculin skin test, compared to only 33.6% of those whose scar formation were not visible ($P<0.05$). Instead, 99.3% of the infants with positive tuberculin skin testing exhibited scar formation compared to 88.0% of those who were negative for tuberculin reactivity ($P<0.05$). The infants with no scars more likely did not respond to tuberculin skin testing compared those with scars ($P<0.05$). The PPD negative subjects were more likely to develop a tiny scar or no scar than the subjects with a positive PPD test ($P<0.05$).

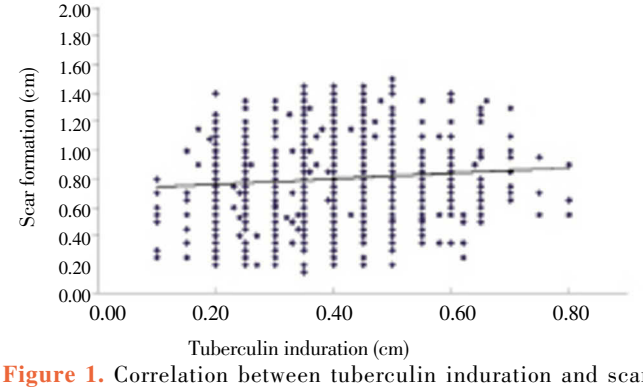

Figure 1. Correlation between tuberculin induration and scar formation.
Correlations between BCG scar formation and tuberculin induration were estimated using Pearson's correlation coefficient, due to the normal distribution. There was a positive correlation ($r= 0.17$, $P<0.05$) between scar formation and tuberculin induration according to all infants ($n=5\ 629$).

Table 1
Status of scar formation and tuberculin testing in different age groups.

Age group	Number (Male/Female)	Frequency of scar formation (%)			Frequency of diameter of tuberculinin induration (%)			Diameter of tuberculinin induration (cm)
		Invisible	≤0.2 cm	>0.2 cm	<0.5 cm	0.5-1.5 cm	≥1.5 cm	
3 months	1 900(1 044/856)	1.7	6.5	91.8	10.1	89.2	0.7	0.776±0.32[a]
6 months	1 875(1 059/816)	2.2	9.4	88.4	10.4	89.2	0.4	0.777±0.30[a]
12 months	1 854(1 011/843)	2.0	5.4	92.6	12.0	87.8	0.2	0.751±0.32[b]
Total	5 629(3 114/2 515)	2.0	7.1	90.9	10.8	88.7	0.5	

According to all infants ($n=5\ 629$), frequencies of scar formation and diameter of tuberculin were calculated. Diameter of tuberculin induration of different age groups is expressed as mean±SD and was analyzed by one-way ANOVA. Means in the last column with different superscript letters are significantly different ($P<0.05$).

3.3. Effect of VA combined VD on in vivo PPD response

Background characteristics were compared between the two intervention groups (Table 2). Sex, mean age, and other background factors of the infants did not differ between the supplementation group and control group. After 3 months, all 597 intervention subjects were returned to give PPD test and check the response. Compararison between the intervention groups is shown in Table 3. The rate of response to PPD was higher in the supplementation group than in the control group ($P<0.05$), and the prevalence ratio of PPD response was 1.07 (95% *CI*: 1.02-1.12). At 15 months of age, 97.4% responded to PPD in the supplementation group, compared to 89.7% in the control group ($P<0.05$), and the prevalence ratio of PPD response was 1.09 (95% *CI*: 1.01-1.17). The males and females in the supplementation group showed higher ratio of PPD response than those in the control group ($P<0.05$) respectively, and the prevalence ratios of PPD response for the supplementation group compared with that for the control group was 1.07 (95% *CI*: 1.01-1.13) for the males and 1.08 (95% *CI*: 1.00-1.17) for the females.

The rate of response to PPD was not different between sexes within the supplementation group and control group respectively (Table 4). For the supplementation group, the mean diameter of tuberculin induration was larger in the females than in the males ($P<0.05$) before the intervention. However, after 3 months of intervention, the males got larger tuberculin induration than the females ($P<0.05$). For the control group, the mean diameter of tuberculin induration was larger in the females than in the males ($P<0.05$) before the intervention, while no significant difference was found between the males and females after 3 months. The interaction analysis between two groups showed no differences in sexes.

4. Discussion

The BCG protective efficacy has been widely reported in Asia[14]. Despite continued efforts have been directed to improve TB control programs at the national level, China remains as a major region with the greatest burden of TB. As TB prevalence rate was high in children[15], BCG vaccination is still the important strategy in China before the appearance

Table 2
Background factors of intervention groups.

Group	Sex percentage (%)		Age percentage (%)			Body length (cm)	Weight (kg)	Head circumference (cm)	BCG scar formation (cm)	DTI[a] (cm)
	Male	Female	3 months	6 months	12 months					
VA combined VD group	53.7	46.3*	28.9*	33.6	37.5	68.1±7.0*	8.9±1.9*	43.9±2.8*	0.31±0.18*	0.19±0.15*
Control group	55.9	44.1	31.4	31.7	36.9	67.9±7.2	9.0±2.0	43.9±2.7	0.31±0.17	0.20±0.19

The background factors except sex and age are expressed as mean±SD. For intervention, 307 infants were enrolled in the supplementation group and 290 in the control group. The subjects in the VA combined VD group received vitamin A (VA) combined vitamin D (VD) drops which contained 1 500 IU VA and 500 IU VD, 1 capsule/d. The background factors of the intervention groups before intervention were analyzed by Student´s *t* test. The sex, mean age and other background factors of the infants did not differ between the supplementation group and control group ($P>0.05$). [a]DIT: Diameter of tuberculinin duration.

Table 3
Prevalence of PPD response in intervention groups after 3-month intervention (%).

Group	Total	Age			Sexes	
		6 months	9 months	15 months	Male	Female
VA combined VD group	96.1(307)*	95.5(89)	95.1(103)	97.4(115)*	97.6(165)*	94.4(162)*
Control group	89.7(290)	89.0(91)	90.2(92)	89.7(107)	91.4(142)	87.5(128)
Prevalence ratio (95% *CI*)	1.07(1.02-1.12)			1.09(1.01-1.17)	1.07(1.01-1.13)	1.08(1.00-1.17)

Chi-square test was used. * indicates significant differences at 0.05 level (vs the control group). The data following the percentages are the number of infants with PPD response.

Table 4
Comparison on PPD response between different sexes of intervention groups before and after intervention.

Group	Sex	Rate of PPD response (%)	Diameter of tuberculin induration (cm)	
			Before intervention	After intervention
VA combined VD group	Male (n=165)	97.6*	0.16±0.15*	0.73±0.21*
	Female (n=142)	94.4	0.23±0.14	0.67±0.20
Control group	Male (n=162)	91.4*	0.17±0.15*	0.72±0.21
	Female (n=128)	87.5	0.23±0.14	0.68±0.22

* indicates significant differences at 0.05 level (*vs* the females in the same group). Diameter of tuberculin induration (mean±SD) was analyzed between the sexes by Student´s *t* test.

of more efficient vaccines. In our study, we found there was a positive correlation between tuberculin induration and scar formation among infants. This finding is similar to a study which confirmed a highly positive correlation between scar formation and vaccination tuberculin sensitivity among preschool children[16]. The size of BCG scar was associated with considerable enhancement in sensitization to tuberculin[17,18]. Studies showed that 98% out of 361 Asian neonates[19] and 95% of 193 Asian vaccinees[20] revealed a positive post-vaccination tuberculin. Conflicting results from the United Kingdom showed that only 45%-46% of vaccinated children were positive in Mantoux when being tested between the ages of 3 months and 2 years[21]. Although the tuberculin state after vaccination was not generally thought to influence the protection offered by BCG, the higher increase of TB was in the mainly unimmunized cohorts born in a European country after 1975, compared with the mainly BCG immunized cohorts born there between 1969 and 1974[22]. It is too difficult to collect blood samples to evaluate immunological activity of infants in large population, because blood collecting is more harmful for infants and low income countries may not afford the cost. We think BCG scar and tuberculin induration are more harmless and visible for evaluation. According to previous study, our findings confirm the trends that the prevalence of PPD positivity was also consistently higher among individuals compared to those without a BCG scar.

In Malawian studies, boys develop scars and PPD responses more frequently than girls[8]. Our investigation showed larger scar formation in the males than in the females in the 6 months group, higher rate of response to PPD test in the males than in the females in the 3 months group, but higher rate of response to PPD test in the females than in the males in the 6 months group. Totally, the rate of all infants was not different between sexes. The relationship between sexes and PPD response is not clear in our study and needs further investigation.

Studies in north and south China showed a high prevalence of VA and VD deficiency among children and infants[23,24]. The high-risk groups were young, dwelled in rural areas with low socioeconomic status, poorly trained doctors and limited medical equipment, had parents with poor education and did not take a regular VA and VD containing supplement. It is associated with high prevalence of TB in rural of China[25]. Epidemiological relationships and largely clinical observation showed a protective role of VD in TB[26]. A study demonstrated that a single dose of 2.5 mg VD for adults significantly enhanced immunity against mycobacteria[27]. Studies from Asia suggested that it might be beneficial to provide VA supplementation at birth to decrease mortality[9,28]. A series of studies in Africa offered high-dose VA (200 000 IU/4 months or 50 000 IU/month) plus BCG vaccination and showed VA supplementation with BCG vaccination does not appear to interfere with the long-term immune response to BCG. VA supplementation at birth can not reduce the mortality and seems to increase the mortality of girls, and diphtheria-tetanus-pertussis vaccinations appeared to affect the rise in retinol binding protein concentrations negatively in girls[11,29]. In our study, we chose 1 500 IU VA and 500 IU VD for 3 months, lower than the tolerable upper intake levels (6 667 IU and 800 IU respectively) and far more lower than the VA provided by studies held in Africa[11,29], so the safety can be insured. According to the above study, many researches depended on VA or VD supplementation and there are both VA and VD deficiency in China, so we chose VA combined VD supplementation in our study. In our findings, the rate of response to PPD was higher in the VA combined VD supplementation group than in the control group. It proved that proper dose of VA and VD supplementation may be beneficial to enhance BCG effects on TB prevention. Two trials in Guinea-Bissau showed that VA supplementation at birth showed no effect and estimated a negative effect on girls and a potentially beneficial effect on boys. There are indications that boys may have a more pronounced Th1 profile than girls since they have a stronger delayed hypersensitivity response[10]. For our supplementation group, the mean diameter of tuberculin induration was larger in the females than in the males before the intervention, but the males got larger tuberculin induration than the females after 3 months of intervention. It indicated that the males may get better effects of TB protection after VA combined VD supplementation.

In conclusion, the correlation between tuberculin induration and scar formation indicated the BCG scar formation may be an useful tool to evaluate the effect of TB prevention. VA combined VD supplementation could raise the rate of PPD positive response and may be beneficial for countries with high prevalence of TB and related VA and VD deficiency. Further study should continue to confirm the effect of VA combined VD in TB prevention by follow-up, longitudinal investigation.

Conflicts of interest statement

All authors declare that they have no conflict of interest.

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