Increased serum antimicrobial peptide LL-37 and HBD-2 combined with 25-hydroxyvitamin D3 deficiency in infants with pertussis

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Abstract

Introduction: Most children with serious infection diseases suffer from malnutrition. Vitamin D participates in the immune response through endogenous antimicrobial peptides (AMPs) regulation. The aim of this study is to investigate the expression of 25-hydroxyvitamin D3 [25(OH)D3], AMPs [LL-37 and human β-defensin 2 (HBD-2)] in the children with pertussis.

Methodology: Serum levels of 25(OH)D3, LL-37, and HBD-2 were detected in 116 children with pertussis aged at 1–12 months (67 males and 49 females). Fifty healthy infants at similar age were employed as normal controls.

Results: The serum 25(OH)D3 levels in the children with mild (27.30 ± 5.98 ng/ml) and severe (24.40 ± 6.27 ng/ml) pertussis were significantly lower than that in the healthy group (30.16 ± 5.13 ng/ml; p <0.01). The vitamin D deficiency rates in children with mild (55.9%) and severe (78.12%) pertussis were significantly higher than that in the control group (34%; p < 0.01). The serum levels of LL-37 and HBD-2 were significantly higher in pertussis patients. Spearman rank correlation analysis did not show any correlation of 25-(OH)D3 with LL-37 or HBD-2.

Conclusions: Most children with pertussis had vitamin D deficiency accompanied by elevated serum LL-37 and HBD-2 levels. However, the average level of 25(OH)D3 at 26.50 ng/ml in the infants with pertussis may not affect the immuno-regulatory ability; thus, the infants with pertussis still maintained a higher level of AMPs (LL-37 and HBD-2) against pertussis infection.

Key words: Vitamin D; pertussis; antimicrobial peptides LL-37; human β-defensin.

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Introduction

Pertussis is an acute and highly infectious respiratory disease caused by Bordetella pertussis, a Gram-negative bacterium. The pathogenic toxins of Bordetella pertussis (including pertussis toxin, tracheal cytotoxin, adenylate cyclase toxin, heat-resistant toxin and endotoxin) can damage and degenerate the ciliated epithelial cells of the trachea, inducing necrosis. The typical manifestation is paroxysmal and spastic cough, i.e., cough at the end of a long chicken crow inspiratory roar (so called whooping cough). The course of the disease is usually as long as 2–3 months. In the early and middle of the last century, pertussis became one of the major diseases seriously threatening children's lives. Since the world-wide implementation of pertussis vaccine immunization, the incidence of pertussis has been sharply reduced. However, in the last decade, the incidence of pertussis has been increasing in many countries and regions, even at the level of outbreaks and epidemics, creating the so called “pertussis recurrence” [1]. The latest data from the World Health Organization in 2018 showed that 151,074 cases of pertussis were reported globally, most of which were in developing countries, and young infants were still the most susceptible population [2]. Due to their lack of complete immunity against Bordetella pertussis, infants represent the population with the highest incidence, hospitalization rate, and mortality caused by pertussis infection. Thus, pertussis has become a contagious disease of widespread concern for pediatric clinicians.

Endogenous antimicrobial peptides (AMPs) are a group of polypeptide substances induced in vivo, which have broad-spectrum and high-efficiency antimicrobial activity [3]. The nuclear acid length of AMPs ranges from 2000-7000 bp, and each consists of 20–60 amino acid residues [4]. Research to date has shown that some AMPs have strong killing effects against some fungi,
protozoa, viruses and even cancer cells [4]. AMPs directly trigger specific immune cells and release chemokines, prompting immune cells to reach the infection site. In addition, AMPs can neutralize endotoxins and inhibit the production and release of inflammatory factors [such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6] through its immunomodulatory effects [3]. Therefore, many scholars tend to refer to these active peptides as polypeptide antibiotics. Natural AMPs also possess selective immune activation and regulative functions, which have shown good preventive and protective effects in sepsis [4]. Moreover, AMPs are widely expressed in many tissues of the body and believed to be an important part of innate immunity and the first line of defense against bacterial infection [3].

Vitamin D mediates host expression of endogenous AMPs through the Toll-like receptor (TLR) pathway, participating in the elimination of invading pathogens and promoting the immune response (25). AMPs mediated by vitamin D mainly include human cathelicidin and β-defensin (HBD) [5]. Increasing clinical evidence indicates that the incidence of infection is higher in people with vitamin D deficiency, whereas supplementation of vitamin D can reduce the incidence of infection [6-8]. Most serious infection diseases are usually accompanied by different degrees of malnutrition. The aim of this study was to investigate whether vitamin D and endogenous AMP levels are abnormal in pertussis infection. We prospectively detected the serum levels of 25-hydroxyvitamin D3 [25(OH)D3] and AMPs (including LL-37 and HBD-2) in children with pertussis of varying severity to analyze their clinical significance.

Methodology

Patients and clinical information

The study participants were initially selected from 122 infants with pertussis, of which 6 cases were excluded due to incomplete data. Thus, a final 116 infants with pertussis 12 months of age or younger (67 males and 49 females) and 50 healthy infants at similar age (27 males and 23 females) were recruited for this study between October 2017 and May 2019 in Yuying Children's Hospital Affiliated to Wenzhou Medical University.

Demographics and other information of the patients were collected, including age, gender, feeding history, sunshine exposure time, feeding method (e.g., breast milk, formula milk or mixed feeding), and duration of direct sunshine (h/wk) exposure in a week. Vitamin D supplementation was recorded as well, including the dose and duration of vitamin D intake. Infants who had a minimum daily intake of 400 IU (international units) of vitamin D for > 1 month were defined as the “vitamin D supplement group”.

Ethical approval

This study was approved by the Ethics Committee of Yuying Children’s Hospital affiliated to Wenzhou Medical University, and informed consent was obtained from patients’ guardians.

Diagnosis of pertussis and exclusion criteria

The diagnosis of pertussis referred to the criteria of the World Health Organization and the United States Centers for Disease Control and Prevention [9,10]. Suspected clinical cases with a positive result from Bordetella pertussis culture or polymerase chain reaction (PCR) detection were diagnosed as pertussis. Cases with recurrent apnea, hypoxemia, pertussis encephalopathy, or cardiovascular dysfunction were considered severe pertussis, and all others were categorized as mild pertussis. The exclusion criteria were: (1) previous diagnosis of vitamin D deficiency or current use of high-dose vitamin D supplementation; (2) recent use of glucocorticoids or other drugs affecting vitamin D metabolism; (3) parathyroid dysfunction, immunodeficiency, rickets, diabetes, severe liver or kidney impairment, or other diseases affecting vitamin D metabolism; and (4) pertussis due to infection by other pathogens. Vitamin D deficiency was defined as a serum 25(OH)D3 concentration below 30 ng/mL.

Pathogen detection

Nasopharyngeal swabs were collected after admission. Bacterial culture was carried out with part of nasopharyngeal swab bedside inoculated with charcoal culture medium. Fluorescent PCR was performed with a B. pertussis nucleic acid detection kit (Shanghai Zhejiang Biotechnology, Zhejiang, China). In brief, total RNA was extracted from another part of the nasopharyngeal swab specimen, reverse-transcribed into cDNA, and amplified by real-time PCR following the instructions of the kit.

Detection of serum 25-(OH)D3, LL-37 and HBD-2

Samples of 3 ml venous blood were collected for serum separation by centrifugation immediately. Serum 25(OH)D3 levels were determined using a chemiluminescent enzyme-labeled immunoassay kit (Roche Diagnostics GmbH, Mannheim, Germany). Endogenous AMP, LL-37 (Hycult Biotech Company,
USA) and HBD-2 protein (Reddot Biotech Company, Canada) levels were determined by enzyme-linked immunosorbent assay (ELISA) according to the definition of the clinical practice guideline of the American Endocrine Society [11].

**Statistical analysis**

Statistical analyses were performed using SPSS 20.0 software (IBM-SPSS, Chicago, IL, USA). Count data were analyzed by χ² test. Measurement data were expressed as mean ± standard deviation (x ± s). Comparisons between the pertussis and control groups were analyzed using independent sample t test. Mean values for multiple groups were compared by one-way analysis of variance. Indices that showed a statistical difference on one-way analysis were further compared between two groups using Fisher's Least Significance Difference (LSD) test. Data with a non-normal distribution or uneven variance were analyzed using the rank sum test. Correlations between LL-37 and HBD-2 with 25-(OH)D were analyzed using Spearman rank correlation. P < 0.05 was considered as statistically significant.

**Results**

**Clinical data**

The average ages of the pertussis patients and the control participants were 5.59 ± 2.62 months and 5.86 ± 2.84 months, respectively. There were no significant differences in gender and age between the two groups (p > 0.05). Among them, 56.03% of the pertussis patients and 66.00% of healthy controls received exclusive breastfeeding (p > 0.05), and 74.14% of patients and 82.00% of controls took vitamin D (400 IU/day) for more than 1 month (p > 0.05). The duration of sun exposure in the pertussis group (2.84 ± 1.43 h/wk) was significantly lower than that in the healthy control group (3.53 ± 1.31 h/wk, P=0.03) (Table 1).

**Serum 25(OH)D3**

Serum 25(OH)D3 in the mild (27.30 ± 5.98 ng/ml) and severe (24.40 ± 6.27 ng/ml) pertussis groups were significantly lower than that in the normal control group (30.16 ± 5.13 ng/ml; p < 0.01). The level of 25(OH)D3 in the severe pertussis group was obviously lower than that in the mild pertussis group (p < 0.05) as well. Overall, 34% of the healthy group was 25(OH)D3 deficient, while 55.95% and 78.12% of infants in the mild and severe pertussis groups had 25(OH)D deficiency, respectively. Thus, the rate of 25(OH)D deficiency in either the mild or severe pertussis group was significantly higher than that in the normal control group (p < 0.01). The percentages of the infants with a serum 25(OH)D level > 20 ng/ml in the severe and mild pertussis groups were 37.50% and 17.86%, respectively, which were significantly higher than that in the normal control group (8%, p < 0.01) (Table 2).

**Serum LL-37 and HBD-2**

The serum LL-37 levels in the pertussis groups were higher than that in the healthy control group (p <0.01), and that in the severe pertussis group was higher than that in the mild pertussis group (p <0.01) (upper panels of Figure 1).

On the other hand, the serum HBD-2 levels in both pertussis groups were significantly higher than that in the healthy control group (p < 0.01). However, the serum HBD-2 level in the severe pertussis group was obviously lower than that in the mild pertussis group (p < 0.05) (lower panels of Figure 1).

| Table 1. Clinical data of the participants. |
|-------------------------------------------|
| Pertussis patients (n = 116) | Healthy controls (n = 50) |
|-------------------------------------------|
| Female/male | 49/67 | 23/27 |
| Age (months) | 5.59 ± 2.62 | 5.86 ± 2.84 |
| Exposure time to sunshine (h/wk)* | 2.84 ± 1.43 | 3.53 ± 1.31 |
| Exclusive breastfeeding (%) | 56.03 | 66.00 |
| Vitamin D supplementation (%) | 74.14 | 82.00 |
| *P < 0.05. |

| Table 2. Serum 25(OH)D3 levels in different groups. |
|-------------------------------------------|
| Pertussis patients (n = 116) | Healthy controls (n = 50) |
|-------------------------------------------|
| 25(OH)D3(ng/ml)* | 27.30 ± 5.98 | 24.40 ± 6.27 | 30.16 ± 5.13 |
| > 30 ng/ml, n (%) | 37 (44.05) | 7 (21.88) | 33 (66) |
| 20-29.9 ng/ml, n (%) | 32 (38.09) | 13 (40.62) | 13 (26) |
| < 20 ng/ml, n (%) | 15 (17.86) | 12 (37.50) | 4 (8) |
| *P < 0.05, healthy control vs. mild or severe pertussis group. |
Correlation of serum LL-37 or HBD-2 level with serum 25-(OH)D3 level

Spearman rank correlation analysis did not show any statistical correlation between LL-37 or HBD-2 expression and the serum 25-(OH)D level (data not shown).

Discussion

Vitamin D is a fat-soluble vitamin that cannot be synthesized by humans independently. Instead, it must obtain through exposure to sunlight or from daily food intake. 25(OH)D3, the main storage form of vitamin D, has a long half-life (2–3 weeks) in the body and is the best indicator of vitamin D levels in living organisms. It has been verified that the major bioactivity of vitamin D is to regulate the metabolic balance of calcium and phosphorus in the body, in order to maintain bone health. With the finding that vitamin D receptors are expressed in various immune cells, vitamin D is believed to play a role in signal conditioning between innate immunity and acquired immunity [12]. The incidence of vitamin D deficiency is high worldwide and in areas both with and without sufficient sunshine. However, because standard diagnosis guidelines for vitamin D deficiency are lacking, different cut-off values are applied around the world for vitamin D deficiency diagnosis. Studies based on community surveys in the past decade in India indicated a prevalence of vitamin D deficiency (< 20 ng/ml of serum 25(OH) D) ranging from 50%–94% [13]. A 2015 investigation in Qujing, Yunnan, China, an area with sufficient sunshine, showed that the average serum 25(OH)D level was 12.08 ± 5.92 ng/ml. Most study participants, at all ages, were vitamin D deficient based on the Chinese cut-off criteria of 30.25 ng/mL [14]. In the present study, the average serum 25(OH)D level was 30.16 ± 5.13 ng/ml in healthy infants in the Wenzhou area of China, which was higher than that in the general population (all age ranges) in Qujing, Yunnan, even through it is lower than the Chinese cut-off level for vitamin D deficiency (30.25 ng/ml). Such a large difference may be caused by different economic levels between the two areas. Our data showed that by using cut-off value from India (< 20 ng/ml), only 8% of the infants in this study were defined as vitamin D deficient.

The infection rate of the pertussis bacterium is about 1–10% in the general population; however, the prevalence of pertussis is only 0.01–0.1%. Thus, Han et al performed a single-nucleotide polymorphism (SNP) study to analyze the genotype of mannose-binding lectin 2, IL-17A, TNFα, vitamin D receptor (VDR), and IL-10 in Dutch patients with pertussis. They found that VDR gene (rs10735810) expression is correlated with pertussis [15]. Interestingly, our data verified this finding in a clinical investigation showing that the serum 25(OH)D3 level of pertussis patients was significantly lower than that of the healthy control group. The vitamin D deficiency rate among children with pertussis was higher than that in the control group, and the serum 25(OH)D3 level of the severe pertussis group was lower than that of the mild pertussis group. Our data indicated that less breastfeeding, less vitamin D supplementation, and less sunshine exposure might be the causes of vitamin D deficiency. Clinical studies verified that the AMP LL-37 has antibacterial activity and is significantly elevated in many infectious conditions, including bacterial pneumonia [16] and urinary tract infections [17]. LL-37 also has been found to have a variety of other biological activities, such as pro-inflammatory and pro-chemotaxis activities, pro-apoptosis, pro-proliferation, and repair of cellular damage [18]. LL37 expression is significantly elevated in the sputum and alveolar lavage fluid in patients with chronic obstructive pulmonary disease [19]. It regulates mucin gene expression via the mitogen-activated protein kinase pathway [20], suggesting that synthesis and secretion of mucin helps abnormally elevation of LL-37. Therefore, LL-37 participates in the process of inflammation and damage repair to protect against the...
invasion of pathogenic microorganisms. Our results showed that the serum LL-37 levels in the patients with mild and severe pertussis were significantly higher than that in the healthy control group. Moreover, the severity of pertussis was closely and positively correlated with the level of serum LL-37. Similar to the results above, the elevation of LL-37 in the infants with pertussis might reflect the innate immune response against pathogenic microorganisms.

HBD-2 is another important factor of the innate immune system. In vitro studies have verified that HBD-2 can play an antibiotic-like role to directly destroy pathogens, including bacteria, fungi, viruses, etc., acting as a first line of defense against respiratory infections [21]. HBD-2 expression was confirmed in lung tissue and found to be significantly elevated in bacterial pneumonia and to decrease after antibiotic treatment [22]. Elahi et al found that newborn piglets were susceptible to infection with Bordetella pertussis, whereas, pigs over 4 weeks of age were not. Further research indicated that the pigs over 4 weeks’ age expressed enough porcine ß-defensin 1 (pBD-1), and supplementation of 500 µg pBD-1 in the newborn piglets significantly decreased the infection rate of B. pertussis. Elahi et al. concluded that host defensins act as an important factor to protect against pertussis through modulation of the innate immune system to combat respiratory infections [23]. Our data indicated that the serum HBD-2 levels in the mild and severe pertussis group were significantly elevated compared with that in the healthy control group. Similar to LL-37, the increased serum HBD-2 level in the infants with pertussis may reflect the innate immune response against pertussis infection. Interestingly, the level of HBD-2 in the severe pertussis group was lower than that in the mild pertussis group. We speculate the possible reason might be that pertussis can produce a variety of virulence factors, among which pertussis toxin has an immunosuppressive effect [24]. Further and severe infection may deteriorate and inhibit immune function, resulting in a decrease in HBD-2 secretion by innate immune cells.

Vitamin D mediates host regulation of human AMP LL-37 and BHD-2 expression through the Toll-like receptor pathway, a process that relies on sufficient 25(OH)D3 levels in the circulation. Insufficiency of 25(OH)D3 will result in an abnormal adjustment process [5,25]. Currently, it is believed that at least 10 ng/ml 25(OH)D3 is necessary for promoting bone and calcium metabolism in the human body, while 20–50 ng/ml can play an immunomodulatory role [6]. In our observation, the average serum 25(OH)D3 level was 26.50 ng/ml in the groups of pertussis patients. A possible explanation is that because the 25(OH)D3 level was above 20 ng/ml, which did not affect the innate immuno-regulatory ability, most infants still maintained elevated AMP levels against pertussis infection.

Conclusion
The incidence of vitamin D deficiency was high in the infant patients with pertussis. However, the average level of 25(OH)D3 at 26.50 ng/mL may not affect the immuno-regulatory ability; thus, the infants with pertussis maintained an increased level of AMPs (LL-37 and HBD-2) against pertussis infection.

Authors’ contributions
SC, WY, ZW, ZX, YC and LJ conceived and designed research; SC, WY and ZW collected data and conducted research; YC, ZX and LJ analyzed and interpreted data; SC and WY wrote the initial paper; YC and LJ revised the paper; LJ had primary responsibility for final content. All authors read and approved the final manuscript.

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