Inflammation, Vol. 37, No. 3, June 2014 (© 2013)
DOI: 10.1007/s10753-013-9790-0

Pharmacological and Pharmacokinetic Studies with Vitamin D-loaded Nanoemulsions in Asthma Model

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Abstract—Vitamin D (VD) was studied for its anti-inflammatory activities with prepared VD-loaded nanoemulsions (VDNM) in ovalbumin-induced asthmatic mice in this paper. In this study, we prepared VDNM for the delivery of VD from the established composition of solid self-emulsifying drug delivery systems (sSEDDS) by spray-drying technique and evaluated its bioavailability (BA) and anti-inflammatory activities in experimental allergic asthma. After the mice were treated orally with VD or VDNM, the plasma 25(OH) D levels, polymorphonuclear cells, tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), total antioxidant activity, and C3 and C4 complement protein levels were studied, respectively. Treatment with VDNM reduced MPO activity, oxidative stress, C3 protein level, O_2^- level as well as the production of IL-1β and TNF-α. Pharmacokinetic studies showed that a significant increase in the maximum concentration (C_{max}) and $AUC_{0\rightarrow 24\ h}$ were observed in VDNM group when compared with VD group ($P<0.01$). The result revealed that VDNM led to an improvement in oral BA of VD in a murine ovalbumin-induced asthma model. These data provided an important proof that VDNM might be a new potential therapy for the management of asthma in humans.

KEY WORDS: asthma; vitamin D; nanoemulsions; bioavailability; inflammatory mediators.

INTRODUCTION

Asthma is a chronic disease associated with airway hyperresponsiveness, airway obstruction, airway remodeling [1, 2]. One of the principal pathophysiological derangements in asthma is chronic inflammation of the lower respiratory tract [3]. Anti-inflammatory agents, such as inhaled steroids and leukotriene receptor antagonists along with long-acting bronchodilators, are the mainstay of asthma pharmacotherapy. However, long-term side effects, prohibitive costs, and suboptimal adherence to asthma medications are ongoing challenges to optimal asthma control. Treatment options are therefore quite limited for asthma and the need to search for other therapies has been recognized by many experts in the field [4, 5].

Vitamin D (VD) is a fat-soluble vitamin which is absorbed in the small intestine and incorporated into chylomicrons. There is growing evidence that VD has a role in the allergic airway diseases [6]. In a recent study, children with severe therapy-resistant asthma that had low VD levels had increased airway smooth muscle mass, worse asthma control and lung function [7]. VD deficiency leads to decreased lung volume, decreased lung function, and altered lung structure [8]. VD supplementation could be a low-cost, practical method to protect groups of people with high incidence of asthma. However, the clinical advancement of VD is hampered by its hydrophobic and highly lipophilic properties result in low oral bioavailability (BA). Therefore, suitable formulation approaches need to be developed to improve the solubility and BA of this poorly soluble compound.

Nanoemulsions (NMs) have reasonable success in improving oral BA of poorly soluble drugs [9, 10]. NMs are colloidal dispersions comprising two immiscible liquids, one of which is dispersed in the other, with droplet sizes between 20 and 200 nm [11]. These fine droplets of emulsions have the advantage of presenting the drug in dissolved form with a large interfacial surface area for drug absorption, which results in enhanced more uniform and reproducible BA [11, 12]. We hypothesized that NM formation will result in enhanced oral BA of VD which then result in an improvement in its pharmacological activity. In

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this study, we prepared VDNM for the delivery of VD from the established composition of solid self-emulsifying drug delivery systems by spray-drying technique and evaluated its BA and anti-inflammatory activities in experimental asthma.

MATERIAL AND METHODS

Preparation of VDNM

Based on the pilot studies, the blank self-emulsifying drug delivery systems (SEDDS) were prepared by mixing of 30 % Miglyol® 812 (oil), 60 % Cremophor® RH40 and Tween80 (surfactant, 2:1) and 10 % Transcutol® P (cosurfactant) at 50 °C with a magnetic stirrer. Then VD and Aerosil 200 (1,000 mg) suspended in 150 ml ethanol were dissolved in the blank SEDDS with stirring until forming an isotropic mixture. The mixture was then kept at room temperature and equilibrating for 24 h. Then it spray dried with a ZPG mini spray dryer B-190 apparatus (Changzhou, China) under the following conditions: inlet temperature 60 °C, outlet temperature 35 °C, aspiration 85 %, feeding rate of the suspension 5 ml/min. The final drug content of the solid NMs was 5.33 % w/w ratio [13].

Animals

Female BALB/c mice (20–22 days old), weighing 12–15 g, were obtained from the Experimental Animal Center of Zhejiang. Mice were housed with free access to food and water in a room with an ambient temperature of 22±2 °C and a 12:12 h light/dark cycle. All experiments were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of Model of Asthma

Mice were sensitized via two intraperitoneal injections of 10 μg of ovalbumin (grade V, ≥98 % pure, Sigma, St. Louis, MO, USA) with alum adjuvant on days 0 and 14 of the experiment. Starting on day 21, the mice, housed in whole-body exposure chambers, were exposed to 1 % aerosolized ovalbumin for 30 min a day and 3 days a week for 9 weeks. The temperature was kept at 20–25 °C and the relative humidity at 40–60 %.

Experimental Design

Mice were randomly assigned to four groups: control group with saline treatment (saline group), asthmatic group with saline treatment (asthmatic group), asthmatic group treated with VD (asthma–VD with a dose of 2,000 IU/kg of VD), and asthmatic group treated with VDNM (asthma–VDNM with a dose equivalent to 2,000 IU/kg of VD). Body weight of the animals was recorded initially at weekly intervals and at autopsy. All animals surviving after 30 days were killed. The blood samples were drawn from orbital vein from all the groups and serum was separated for biochemical estimations. Tissue samples of thymus and spleen were dissected from the visceral tissues. After washing with saline, the tissue samples were blotted dry and weighed. All samples were stored at −80 °C for future analysis.

Pharmacokinetic Studies of VDNM

Pharmacokinetic parameters were calculated from the corresponding plasma concentration–time curves using noncompartmental analysis (WinNonlin 4.0, Pharsight, Mountain View, CA, USA). The area under the plasma concentration–time curve from time 0 to time infinity ($AUC_{0-\infty}$), maximum concentration (C_{max}), and time to reach C_{max} (T_{max}) were determined from the concentration–time profile.

Measurement of MPO Activity

Myeloperoxidase (MPO) activity was measured to assess the extent of polymorphonuclear cell (PMN) infiltration into the plasma using a commercial assay kit (Nanjing Jiancheng Bioengineering, China).

Measurement of IL-1β and TNF-α

The concentration of interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in the plasma was determined using a commercial ELISA kit (Shanghai Jinma Biological Technology, China) according to the manufacturer's instructions.

Measurement of TAOS

The total antioxidant status (TAOS) of the supernatant of centrifuged plasma was determined by the way introduced by Laight *et al.* [14]. The increase in absorbance at 405 nm was measured by using a microplate reader (Shanghai Xunda Medical Technology, China).

Measurement of C3 and C4 Complement Proteins

The serum complements C3 and C4 were measured using standard diagnostic tests and a semi-auto

Biochemical Analyzer (SBA-733, Sunostik Medical Biotechnology, Jilin, China).

Measurement of Immune Organ

The impact of VDNM on immune organ was evaluated based on the thymus index and spleen index. The thymus or spleen index was calculated by the following formula: thymus (spleen) index=weight of thymus (spleen) (mg)/weight of mouse (g).

Data Analysis

All data were analyzed by a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test. The data represents means and standard error. The significant level of 5 % ($P<0.05$) was used as the minimum acceptable probability for the difference between the means.

RESULTS

Pharmacokinetic Parameters of VDNM

In the present study, an *in vivo* absorption study was undertaken to determine whether or not the VDNM could increase the GI absorption of drug after oral administration. As measurement of serum 25(OH) D is widely accepted to be the most useful marker for assessment of the individual VD status, Table 1 shows the pharmacokinetic variables of 25(OH) D measured in this study. The total plasma concentration of 25(OH) D after oral administration of VD is 26.28±7.5 ng/ml. However, the plasma level of 25(OH) D in the VDNM group is 38.30±9.1 ng/ml. A significant increase ($P<0.001$) in the C_{max} and $AUC_{0\rightarrow24\ h}$ were observed in the VDNM group when compared with the VD group.

Table 1. Pharmacokinetic Parameters after Oral Administration of VDNM to Mice (Each Group)

Groups	$AUC_{0\rightarrow24\ h}$ (ng h/ml)	C_{pmax} (ng/ml)	T_{max} (h)
Asthma–VDNM group (n=10)	73.50±11.1*	38.30±9.1*	3.56±0.23*
Asthma–VD group (n=10)	55.33±9.0	26.28±7.5	5.22±0.30

Values are shown as means±SEM
* $P<0.01$

Effects of VDNM on Activity of MPO

The activity of MPO in the blood samples was measured as an indicator of PMN migration. The results showed that MPO activity was relatively low in the saline group, but significantly increased in the ovalbumin-induced asthma group. Treatment with VDNM significantly reduced ovalbumin-induced MPO activity compared with that in the asthmatic group ($P<0.01$, Table 2). VD treatment also reduced ovalbumin-induced MPO activity; however, this was not statistically significant.

Effects of VDNM on Inflammatory Mediators

Fig. 1 showed that the level of inflammatory cytokines were relatively low in the saline group, ovalbumin-induced asthma significantly increased protein concentration of IL-1β in the blood (Fig. 1 A). VDNM and VD treatment decreased the level of IL-1β as compared to the asthmatic group respectively ($P<0.01$, $P<0.05$). As shown in Fig. 1 B, TNF-α levels were significantly higher in the ovalbumin-induced asthma group as compared with saline group. VDNM suppressed this response ($P<0.05$).However the same result did not occur in the VD-treated group.

Effects of VDNM on TAOS

The saline group had a lower level of total antioxidant activity (TAOS) activity. However, it significantly increased in the ovalbumin-induced asthma group ($P<0.01$; Fig. 2). VDNM suppressed this response ($P<0.05$). Treatment with VD also reduced level of TAOS activity; however, this was not statistically significant ($P>0.05$).

Effects of VDNM on C3 and C4 Complement Proteins

The C3 value was 0 in the saline group, a significant rise in C3 in the asthmatic group relative to the saline group is observed. However, C3 drops in the VDNM group in the inflammatory reaction (Table 3). On the contrary, the C4 value was 7.7±0.4 mg/dl; it drops in the asthmatic group

Table 2. Effects of VDNM and VD on MPO Activity

Groups	U/g^{-1}
Saline group	0.98±0.20*
Asthmatic group	2.10±0.20
Asthma–VDNM group	1.10±0.11*
Asthma–VD group	1.88±0.22

Values represent the mean ± SEM.
* $P<0.05$ vs. asthmatic group

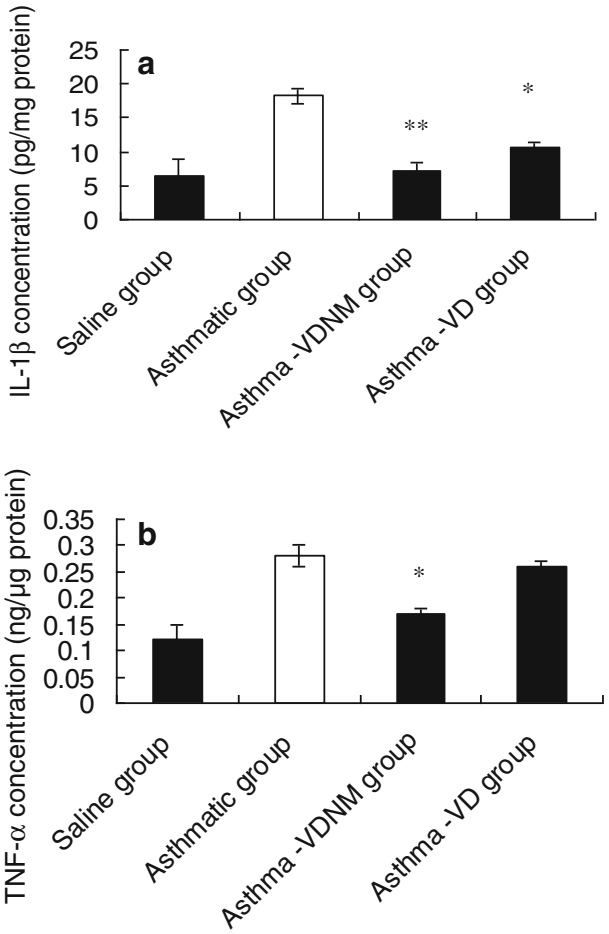

Fig. 1. Effect of VDNM and VD on inflammatory cytokines. Values are shown as means±SEM. *P<0.05 vs. asthmatic group, **P<0.01 vs. asthmatic group.

(P<0.01). However, statistical analysis did not demonstrate an essential difference in the variation of both VDNM and VD group (Table 3).

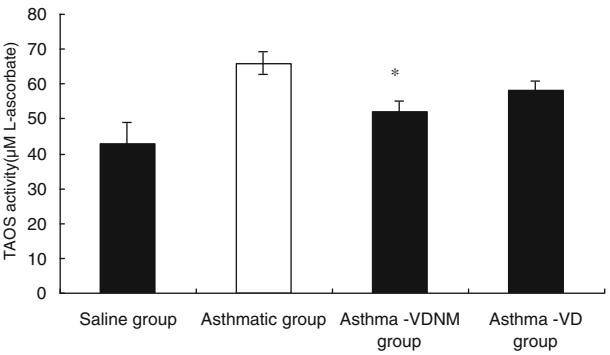

Fig. 2. Effect of VDNM and VD on total antioxidant activity. Values are shown as means±SEM. *P<0.05 vs. asthmatic group.

Table 3. Effect of VDNM on the C3 and C4 Complement Proteins

Different groups	C3 (mg/dl)	C4 (mg/dl)
Saline group	0	7.7±0.4
Asthmatic group	52.0±3.0	3.8±0.7
Asthma–VDNM group	19.6±1.1**	4.6±0.6
Asthma–VD group	46.7±2.2	4.4±0.3

Values are shown as means±SEM
** P<0.01 vs. asthmatic group

Impact of VDNM on Immune Organ

Table 4 shows that the thymus index and spleen index of mice in saline group were 2.672±0.230 and 8.043±0.296, respectively. On the contrary, the two indexes were decreased significantly in the asthmatic group (P<0.05). VDNM suppressed this response (P<0.05). However, there was no significant difference between the asthmatic group and the VD-treated group (Table 4).

DISCUSSION

It is generally accepted that asthma is caused by an inappropriate, Th2-based immune response to environmental allergens in genetically predisposed individuals [15]. The need to search for new therapies for asthma has been recognized by many experts in the field. Recent studies have provided strong evidences that VD had a significant role in both direct and indirect regulation of proliferation, differentiation and function of immune cells [16]. Also, the anti-inflammatory effect of VD was recognized by inducition of FoxP3, a commitment transcription factor for Treg lymphocytes [17–19]. It binds to its receptor, VD receptor, which is a transcription factor that interacts with its co-regulators and alters the transcription of target gene involved in a wide spectrum of biological responses [19]. However, the clinical advancement of VD is hampered by its hydrophobic and highly lipophilic

Table 4. The Effect of VDNM and VD on Immune Function

Different groups	Thymus index (×10^3)	Spleen index (×10^3)
Saline group	2.672±0.230*	8.043±0.296*
Asthmatic group	1.739±0.980	5.179±0.561
Asthma–VDNM group	2.660±0.066*	7.788±0.180*
Asthma–VD group	2.162±0.090	5.843±0.336

Values are shown as means±SEM
* P<0.05 vs. asthmatic group

properties result in low oral BA. To overcome this problem, NMs are being investigated as potential new colloidal carrier for lipophilic drugs such as VD. In this study, the VDNM was studied for its anti-inflammatory activities.

The main reason for the enhanced drug oral BA by NM is the excellent efficiency of SEDDS in improving the drug solubility and increasing the dissolution rate [20, 21]. In the present study, an *in vivo* absorption study was undertaken to determine whether or not the enhanced solubility and *in vitro* dissolution of VD in a VDNM could increase the gastro-intestinal (GI) absorption of drug after oral administration. The results suggested that oral VD absorption in the VDNM was significantly increased compared to that of the VD formulation. The current data were consistent with the findings of many other poor solubility drugs [22, 23]. This increase in BA will result in enhanced pharmacological activity. The anti-inflammatory and antioxidant assay revealed that the therapeutic efficiency of VD significantly enhanced upon NM formation.

Adequate assessment of inflammatory cells, cytokines, chemokines, and antiiflammatory molecules is essential for understanding, monitoring, and treating lung diseases. Among these inflammatory mediators, PMN infiltration, IL-1β and TNF-α are of particular importance because they play a major role in coordinating mechanisms that command pro-inflammation. The suppression of these pro-inflammatory mediators had been found to reduce the severity of the inflammatory reaction [24]. We found that VDNM significantly reduced the PMN infiltration and levels of IL-1β and TNF-α. However, the same result did not occur in the VD-treated group. These studies supported our hypothesis that VDNM may enhance VD anti-inflammatory effects.

The lungs of asthmatic patients are exposed to oxidative stress due to the generation of reactive oxygen as a consequence of chronic airway inflammation. The available evidence tends to support the concept that the oxidant/antioxidant equilibrium is disturbed in asthma [25]. The TAOS is an indication of O_2^- and other oxidant species. We measured TAOS activity as an indirect indication of the formation of O_2^- and other oxidant species. This index was increased in the asthmatic group induced by ovalbumin in comparison with the control group. In contrast, the TAOS activities of the VDNM group were lower than those of asthmatic group, suggesting that O_2^- generation was inhibited by the VDNM formation.

C3 and C4 proteins are complement and acute phase proteins. The concentration of these proteins changes during inflammation and tissue damage. The C3 level rises during the inflammatory reaction. The C4 concentration changes in sinusoid way, which is a result of C4 synthesis and consumption processes [26]. In our studies, the level of C3 protein was much higher in asthmatic group than that in the saline group. As one complement proteins, the high C3 level indicated the development of a defense reaction. This reaction is necessary to inhibit the inflammatory process [27]. VDNM significantly reduced the level of C3 protein. We hypothesized that it was the result that inhibit the inflammatory process by VDNM. However, statistical analysis did not demonstrate an essential difference in the variation of VD on the level of C4 protein. It probably needs more hours to show the C4 concentration changes in sinusoid way [26].

Asthma is an immunologic disease characterized by airway inflammation. The main immune organs in mice are the thymus and spleen. During an immune response, mature lymphocytes and other immune cells interact with antigens in these tissues. Consequently, immune tissue mass can, in some cases, indicate immune status [28]. Our data demonstrated that VDNM treatment significantly reduces the trend of atrophy of the spleen and thymus in asthmatic mice. However, VD was not able to reverse the effect of asthma on spleen and thymus indices. These results indicated that VD could not enhance the immune functions without the NM formation.

In conclusion, this study had demonstrated profound differences between the activities of VD and its NM formation in experimental asthma model by using a variety of testing systems. These data provided an important proof that VDNM might be a new potential therapy for the management of asthma in humans.

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