

Vitamin D improves corticosteroid efficacy and attenuates its side-effects in an animal model of asthma

Anita A. Mehta, Ashok D. Agrawal, Vasu Appanna, and Kiranj K. Chaudagar

Abstract: The subacute use of corticosteroids has side-effects such as glucose intolerance, dyslipidemia, anxiety, and depression, which could be halted with vitamin D, which is an immunomodulatory vitamin. Thus, we aimed to study the anti-asthmatic efficacy and side-effects profile of vitamin D, the corticosteroid dexamethasone, and their combination on ovalbumin-induced airway inflammation in rats. For this, 2 different doses of vitamin D (50 IU/kg, daily for 2 weeks, or and 60000 IU/kg, bolus dose, by intraperitoneal injection (i.p.)) were administered in combination with dexamethasone (2.5 mg/kg, i.p., for 2 weeks) prior to challenge with ovalbumin. At the end of the therapy, the asthmatic parameters such as differential white blood cell counts, serum levels of immunoglobulin E, bronchoalveolar lavaged fluid, and interleukin-5, as well as serum levels of nitric oxide were significantly increased after allergen challenges in asthmatic rats as compared with the controls. Such increases were significantly attenuated by monotherapy with vitamin D and with combination therapy of vitamin D and dexamethasone, where the combination therapy was superior to the monotherapy. Dexamethasone-induced hyperglycemia, hyperlipidemia, and behavioral abnormalities in the allergic rats were attenuated with vitamin D. The daily dose was better for controlling serum levels of immunoglobulin E than the bolus dose, whereas the bolus was superior for reducing dexamethasone-induced psychotropic abnormalities. There were no significant changes in other parameters between the daily and the bolus dose. In conclusion, a daily dose of vitamin D in combination with dexamethasone is more efficacious for treating asthma in allergic rats than monotherapy.

Key words: ovalbumin, dexamethasone, single dose, bolus dose.

Résumé : L'utilisation subaiguë de corticostéroïdes provoque des effets secondaires comme l'intolérance au glucose, la dyslipidémie, l'anxiété et la dépression, qui pourraient être contrecarrés par la vitamine D, une vitamine immuno-modulatrice. Les auteurs avaient pour but d'étudier l'efficacité antiasthmatique et le profil des effets secondaires de la vitamine D, de la dexaméthasone et de leur combinaison, dans un modèle d'inflammation des voies respiratoires induite par l'ovalbumine chez le rat. Deux doses différentes de vitamine D (50 UI/kg, i.p., quotidiennement pendant 2 semaines et 60000 UI/kg bolus) ont été administrées en combinaison avec la dexaméthasone (2,5 mg/kg, i.p. pendant 2 semaines) avant une provocation à l'ovalbumine. À la fin de la thérapie, les paramètres asthmatiques comme la numération différentielle des globules blancs, les IgE du sérum et du liquide de lavage broncho-alvéolaire, l'IL-5 et le NO du sérum étaient significativement accrus à la suite de la provocation chez les rats asthmatiques comparativement aux contrôles. Un tel accroissement était significativement atténué par la combinaison ou monothérapie de vitamine D et de dexaméthasone, la thérapie combinée étant supérieure à la monothérapie. L'hyperglycémie, l'hyperlipidémie et les troubles de comportements induits chez les rats allergiques par la dexaméthasone étaient atténués par la vitamine D. La dose quotidienne était plus efficace pour contrôler les IgE que le bolus, alors que le bolus était plus efficace pour réduire les troubles du comportement que la dose quotidienne. Il n'y avait pas de changements significatifs des autres paramètres entre la dose quotidienne et le bolus. En conclusion, une dose quotidienne de vitamine D combinée à la dexaméthasone est plus efficace pour traiter l'asthme chez les rats allergiques que la monothérapie. [Traduit par la Rédaction]

Mots-clés : ovalbumine, dexaméthasone, dose unique, dose bolus.

Introduction

Asthma is one of the most common chronic inflammatory diseases, and is ranked among the top 10 prevalent conditions. It affects about 300 million people worldwide and is projected to increase to 400 million by 2025 (Masoli et al. 2004; Adcock et al. 2008). In 2010, the number of casualties from this disease was more than 300 thousand (Lozano et al. 2013). Asthmatic inflammation is characterized by the activation of mast cells, infiltration of eosinophils, and increased activated T helper 2 (Th2) lymphocytes that express interleukins (IL) 4, 5, and 13 (Barnes and Adcock 1993; Barnes 1995). Current treatments for asthma are based on inhaled cortico-

steroids and long- and short-acting β_2 -adrenoreceptor agonists, as well as leukotriene antagonists. Inhaled corticosteroid has become the first-line treatment for most patients (Barnes and Adcock 2009). However, long-term use of corticosteroids is known to induce various side-effects like hyperglycemia, hypertension, cardiometabolic abnormalities, adverse psychiatric effects, weight gain, and osteoporosis (Moghadam-Kia and Werth 2010). Moreover, chronic use of corticosteroids induces vitamin D deficiency, and low levels of vitamin D contribute to increased asthma severity (Searing et al. 2010).

Bioactive vitamin D (calcitriol) is a steroidal hormone that not only plays an important role in the metabolism of calcium, phos-

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phorus, and bone, but is also involved in the regulation of the immune system. Vitamin D from the skin and diet is metabolized in the liver to 25-hydroxyvitamin D (25(OH) vitamin D), which is further metabolized in the kidneys to its active form, 1,25-dihydroxyvitamin D (1,25(OH)₂D₃). The biological responses to the 1,25(OH)₂D₃ are mediated by vitamin D receptor (VDR), which is a member of the superfamily of nuclear hormone receptors. VDR is expressed by many cells of the immune system, including activated B and T cells, monocytes, and dendritic cells (Bouillon et al. 1995; Dong et al. 2003; Griffin et al. 2003; Chambers and Hawrylowicz 2011). Vitamin D inhibits Th1- (Lemire et al. 1995), Th2- (Topilski et al. 2004), and Th17- (Tang et al. 2009) associated cytokines, whereas there is some evidence that vitamin D may enhance Th2 responses (Lange et al. 2009). Regulatory T-cells (Tregs) are a subpopulation of T cells that modulate the immune system (Hewison 2010), and which exert their effect by inhibiting transcription of the inflammatory cytokine IL-2, expressing IL-10, and potentially converting effector T cells to hyporesponsive or regulatory forms (Shevach 2002; Chambers and Hawrylowicz 2011). Vitamin D causes the induction as well as proliferation of Tregs (Gorman et al. 2007; Chambers and Hawrylowicz 2011) and increased IL-10-secretion and Toll-like receptor (TLR)-9 expression (Urry et al. 2009). Both in vivo and in vitro studies have shown that vitamin D enhances the production of anti-inflammatory cytokine IL-10 by human T cells (Provvedini et al. 1986). Moreover, altered vitamin D homeostasis is associated with increased risk of developing glucose intolerance (Hypponen and Power 2006), metabolic syndrome (Hypponen et al. 2008), cardiovascular events (Cheng et al. 2009), psychiatric effects (Harms et al. 2010), obesity (Konradsen et al. 2008), and hypertension (Forman et al. 2007). There is disparity between the doses of vitamin D that can be administered in a daily dose or as a bolus dose (single high dose) (Bergman et al. 2013).

For this study, we aimed to assess the anti-asthmatic potential and the side-effects profile of a combination treatment with corticosteroid and vitamin D in allergic rats.

Materials and methods

Animals

Male albino Wistar rats (150–200 g) were housed in a climate-controlled room (temperature $22 \pm 1^\circ\text{C}$; relative humidity $55\% \pm 5\%$) on a 12 h (light) – 12 h (dark) cycle. Animals had access to a standard pellet diet (certified Amrut brand rodent feed; Pranav Agro Industries, Pune, India) and filtered tap water ad libitum. All experiments were carried out with strict adherence to ethical guidelines and were conducted according to the protocol (LMCP/Cology/12/12) approved by the Institutional Animal Ethics Committee (IAEC), and according to Indian norms set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, New Delhi, India). Throughout the entire study period, the animals were monitored for growth and health status to be certain that they were healthy.

Reagents

All solvents used in the study were of analytical grade. Diethyl ether was purchased from Rankem (New Delhi, India), ovalbumin (grade V) from Sigma-Aldrich (St. Louis, Missouri, USA), xylazine from Five Star Pharmaceuticals (Ahmedabad, India), vitamin D from Cadila Pharmaceuticals (Ahmedabad, India), dexamethasone from Cadila Healthcare (Ahmedabad, India), and ketamine from Themis Medicare (Goregaon, India). Kits for blood glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were purchased from Span Diagnostics (Gujarat, India). Kits for IL-5 and immunoglobulin E (IgE) were purchased from Pro Lab Marketing, New Delhi, and GenWay Biotech (San Diego, California, USA) respectively.

Sensitization and challenge with ovalbumin and treatment

Animals were distributed among 6 groups ($n = 6$ animals/group). Group I, the non-sensitized controls, received saline (2.5 mL/kg body mass); group II, the model control group, was sensitized with ovalbumin (OVA) and then received saline (2.5 mL/kg); group III, the reference standard group, was sensitized with OVA and then received dexamethasone (Dexa) (2.5 mg/kg); group IV, an experimental group, was sensitized with OVA and then received vitamin D (50 IU/kg); group V, an experimental group, was sensitized with OVA and then received a combination of vitamin D (50 IU/kg) and Dexa (2.5 mg/kg); and group VI, an experimental group, was sensitized with OVA and then received the combination of single high dose (bolus) of vitamin D (60000 IU/kg) and Dexa (2.5 mg/kg). All rats except group I were sensitized with 100 μg OVA (adsorbed on 100 mg/mL of aluminum hydroxide in saline) by intraperitoneal (i.p.) injection on days 0, 14, and 21 (general sensitization). Rats were anesthetized with ether before being challenged with 100 μg of OVA in 100 μL of saline, intra-nasally 4 times/week for 3 weeks (Duan et al. 2003). Vitamin D was dissolved in 96% (v/v) ethanol and freshly diluted in saline before administration. On days 28–45, groups II–V received daily doses of the drug or the vehicle, and group VI received the bolus dose of vitamin D (60000 IU/kg, on day 28) and then Dexa (2.5 mg/kg, days 28–45) given daily by i.p. injection 1 h prior to OVA administration (Ish-Shalom et al. 2008).

Cellular count and serum preparation

On day 46, blood (3 mL) was collected from each animal under light ether anesthesia. Each sample was then divided into 2 portions. The first aliquot (2.5 mL) was placed in a nonheparinized tube for serum separation; the isolated serum was stored at -80°C until quantitative determination of cytokines. Complete blood counts were performed with an automated hematology analyzer (Sysmex XE-2100 Blood Cell Analyzer; Sysmex, Milton Keynes, UK).

Bronchoalveolar lavaged fluid

At the end of the experiment (i.e., on day 46), bronchoalveolar lavaged fluid (BALF) was collected from each animal. An overdose of ketamine (30 mg/kg) and xylazine (20 mg/kg) was administered by subcutaneous (s.c.) injection. A polypropylene cannula (24G) was inserted into the trachea, and then, 0.9% (w/v) normal saline solution (10 mL) was introduced into the lungs via a 10 mL syringe at $37 \pm 1^\circ\text{C}$ and then recovered 5 min later. The recovered BALF (5 mL) was centrifuged at 500g for 10 min at 4°C ; the resulting supernatant was collected and stored at -80°C for cytokine determination.

IL-5 and IgE

The levels of IgE and IL-5 in each sample of recovered serum (400 μL) and BALF (4.5 mL) were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (IL-5, ProLab Marketing; IgE, GenWay Biotech). All plates were analyzed using an automated plate reader (Lab System Multiscan Model-51118220; Thermo Bioanalysis, Helsinki, Finland).

Measurement of nitrites

Nitrites were analyzed by adding 20 μL of freshly prepared Griess reagent (1% sulfanilic acid in 5% H_3PO_4 and 0.1% *N*-1-naphthyl ethylene diamine dihydrochloride) in double-distilled H_2O to 20 μL of serum from each experimental group. Samples were incubated at room temperature for 20 min and spectrophotometric readings were taken at 546 nm. The nitrite concentrations were determined using a linear standard curve between 1 and 150 $\mu\text{mol/L}$ sodium nitrite. Assays were performed in triplicate (Green et al. 1982).

Table 1. Effect of treatments on total cells and differential leukocyte counts in blood ($\times 10^5$ cells/mL).

Treatment groups	Eosinophils	Neutrophils	Lymphocytes	Monocytes	Total cell
Control	0.070 $\pm$ 0.01	0.032 $\pm$ 0.004	1.175 $\pm$ 0.12	0.029 $\pm$ 0.003	2.532 $\pm$ 0.022
S	0.792 $\pm$ 0.06 [†]	0.676 $\pm$ 0.075 [†]	3.969 $\pm$ 0.10 [†]	0.114 $\pm$ 0.010 [†]	6.266 $\pm$ 0.06 [†]
S+Dexa ^a	0.343 $\pm$ 0.02*	0.223 $\pm$ 0.016*	2.117 $\pm$ 0.11*	0.045 $\pm$ 0.007*	3.896 $\pm$ 0.017*
S+VitD ^b	0.421 $\pm$ 0.05*	0.285 $\pm$ 0.039*	2.436 $\pm$ 0.21*	0.049 $\pm$ 0.003*	4.438 $\pm$ 0.34*
S+Dexa ^a +VitD ^b	0.160 $\pm$ 0.04* [#]	0.047 $\pm$ 0.006* [#]	1.168 $\pm$ 0.020* [#]	0.029 $\pm$ 0.005*	2.585 $\pm$ 0.46* [#]
S+Dexa ^a +VitD ^c	0.226 $\pm$ 0.03*	0.069 $\pm$ 0.023* [#]	1.113 $\pm$ 0.07* [#]	0.024 $\pm$ 0.002*	2.439 $\pm$ 0.24* [#]

Note: Values shown are the mean $\pm$ SEM ($n = 6$ rats per group); [†], $p < 0.05$ compared with the non-sensitized control; *, $p < 0.05$ compared with the ovalbumin (OVA) sensitized vehicle-treated control; [#], $p < 0.05$ compared with the dexamethasone-treated group. S, OVA sensitized; VitD, vitamin D; Dexa, dexamethasone.

^aDose of 2.5 mg/kg body mass.

^bDose of 50.0 IU/kg body mass.

^cDose of 60000 IU/kg body mass.

Corticosteroid-induced side-effects study

Biochemical estimations for metabolic side-effects

The blood glucose, total cholesterol, HDL-cholesterol, and tri-glyceride estimations were carried out as per the instructions provided by the manufacturers with the kits. The levels of LDL-C were calculated using the Friedewald equation [$\text{LDL-C} = \text{CT} - (\text{HDL-C} + \text{TG}/5)$] (Friedewald et al. 1972).

Psychiatric side-effects

At the end of the study, the animals were subjected to the following models for assessing psychiatric effects.

Assessment of anxiolytic activity

Open-field test

The open-field area was made of wood (60 cm $\times$ 60 cm $\times$ 40 cm) divided into 16 equal squares. This apparatus was used to evaluate the exploratory activity of the animal. Rats were placed individually in the apparatus. The parameter observed for 5 min was number of squares crossed (with all 4 paws) (Kennett et al. 1985).

Marble burying test

The method of Broekkamp et al. (1986) was used with substantial modification. The test apparatus was a 40 cm $\times$ 25 cm $\times$ 12 cm plastic cage similar to the animal's home cage. The floor of the cage was evenly covered with a 5 cm layer of sawdust, and 16 glass marbles (1.5 cm in diameter) were evenly distributed on the sawdust in the cages. Each rat was assigned to a particular test cage for 30 min and the number of marbles buried at least 2/3 of the way in the sawdust was recorded.

Assessment of antidepressant activity

Tail suspension test

In the tail suspension test, a rat suspended by the tail shows initial struggling, followed by periods of immobility that increase in duration across the 6 min test. The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985) as a means of evaluating potential antidepressants. Rats were hung individually 58 cm above the floor by the adhesive tape placed approximately 1 cm from the tip of the tail. Immobility was recorded during a 6 min period, during which the initial immobility of 2 min was discarded. The animal was considered to be immobile when it did not show any body movement and hung passively.

Statistical analysis

Results are reported as the mean $\pm$ SEM from 6 animals per group. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed with Tukey's post-hoc test; differences were considered statistically significant for values of $p < 0.05$. All statistical analyses were performed using GraphPad software (San Diego, Calif.).

Results

Impact of treatments on body mass, circulating cellular counts, and IgE and IL-5 levels in serum

None of the animals present in the model control (group II) or drug regimen groups (groups III and VI) showed a significant difference in body mass during the experimental period by comparison with the animals in the non-sensitized control group (group I). Furthermore, there were no apparent effects on the appetite/water consumption or on the outward appearance (i.e., fur and eyes) of animals in each treatment group (data not shown).

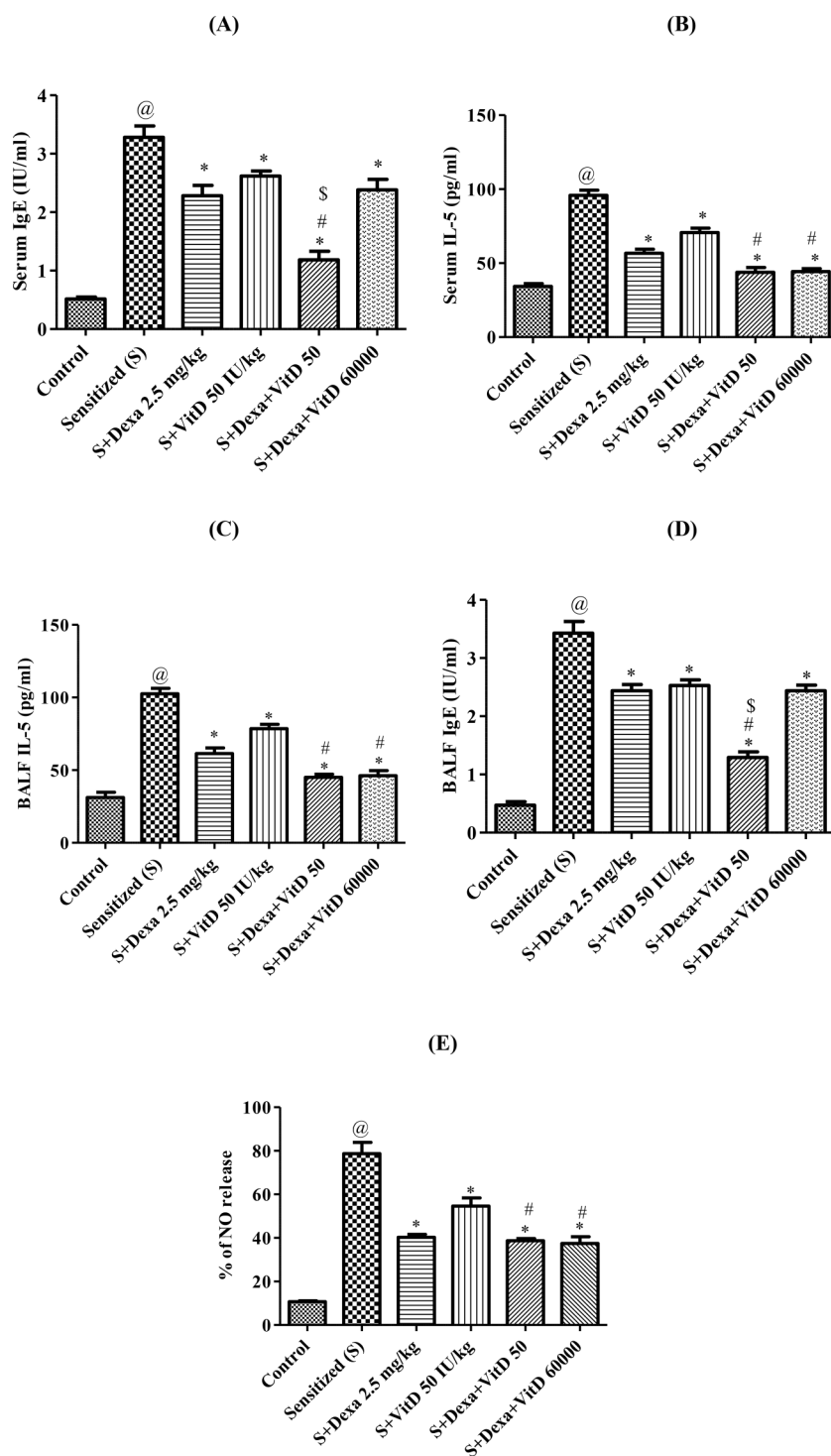
The total number of leukocytes as well as the total number for each type of leucocyte in the blood samples recovered from the model control animals were markedly increased ($p < 0.05$) compared with the non-sensitized controls. However, the numbers of circulating eosinophils ($p < 0.05$), lymphocytes ($p < 0.05$), monocytes ($p < 0.05$), neutrophils ($p < 0.05$), and total cells ($p < 0.05$) in the blood were significantly decreased in the groups treated with Dexa, with the daily dose of 50 IU/kg vitamin D, with the combination of the daily dose of vitamin D (50 IU/kg) and Dexa, or with the bolus dose of vitamin D (60000 IU/kg) and the daily dose of Dexa, compared with those numbers for the OVA-sensitized rats. However, the combination of the daily dose of vitamin D with Dexa and the bolus dose of vitamin D (60000 IU/kg) with the daily dose of Dexa resulted in significantly decreased numbers of circulating eosinophils ($p < 0.05$), lymphocytes ($p < 0.05$), and neutrophils ($p < 0.05$) as compared with the Dexa-treated rats, respectively. There was no significant difference between the groups treated with vitamin D at 50 IU/kg and vitamin D at 60000 IU/kg, with respect to changes in eosinophil, lymphocyte, monocyte, neutrophil, and total cell counts (Table 1).

The control animals showed significant ($p < 0.05$) increases in plasma levels of IgE and IL-5 compared with the non-sensitized controls. These elevated levels of IgE ($p < 0.05$) and IL-5 ($p < 0.05$) were significantly decreased in rats that received Dexa, vitamin D at the daily dose of 50 IU/kg, the combination of a daily dose of vitamin D (50 IU/kg) and Dexa, or the bolus dose of vitamin D at 60000 IU/kg with the daily dose of Dexa, respectively. However, serum levels of IgE and IL-5 were significantly ($p < 0.05$) decreased by the combination of a daily dose of vitamin D with Dexa or the bolus dose of vitamin D (60000 IU/kg) with the daily dose of Dexa (Figs. 1A and 1B) as compared with Dexa, respectively. The low dose of vitamin D (50 IU/kg) significantly ($p < 0.05$) reduced serum IgE as compared with the high dose of vitamin D (60000 IU/kg); however, this was not statistically significant in the case of serum IL-5.

Influence of treatments on IgE and IL-5 in bronchoalveolar lavaged fluid

The significant ($p < 0.05$) increase in cytokine levels in BALF from the control group was not present in the groups treated with Dexa, vitamin D at 50 IU/kg, the combination of a daily dose of

Fig. 1. Effect of treatments on (A) serum levels of immunoglobulin E (IgE), (B) serum levels of interleukin 5 (IL-5), (C) bronchoalveolar lavaged fluid (BALF) levels of IL-5, (D) BALF levels of IgE, and (E) on serum levels of nitric oxide (NO) from male albino Wistar rats. Values shown are the mean $\pm$ SEM ($n = 6$ rats per group); @, $p < 0.05$ compared with the non-sensitized control group; *, $p < 0.05$ compared with the ovalbumin (OVA)-sensitized vehicle-treated model control group; #, $p < 0.05$ compared with the dexamethasone-treated group; §, $p < 0.05$ compared with the group treated with vitamin D at the dose of 60000 IU/kg. S, ovalbumin-sensitized group; VitD, vitamin D; Dexa, dexamethasone.



vitamin D at 50 IU/kg and Dexa, or the bolus dose of vitamin D at 60000 IU/kg with daily dose of Dexa, respectively [IgE ($p < 0.05$) and IL-5 ($p < 0.05$)]. However, the combinations of a daily dose of vitamin D with Dexa or the bolus dose of vitamin D (60000 IU/kg) with a daily dose of Dexa caused a significant ($p < 0.05$) reduction in IL-5 (Fig. 1C) levels by comparison with the Dexa treatment.

In contrast, only the combination of a daily dose of vitamin D with Dexa caused a significant ($p < 0.05$) reduction in IgE (Fig. 1D) levels by comparison with the Dexa treatment. Treatment with vitamin D at the dose of 50 IU/kg produced a significant ($p < 0.05$) decrease in BALF IgE, but not in IL-5, as compared with the dose of 60000 IU/kg.

Effect of treatments on nitric oxide

The model control animals showed significant ($p < 0.05$) increases in NO levels compared with the non-sensitized controls. These elevated levels of NO ($p < 0.05$) were significantly decreased in rats that received Dexa, vitamin D at the daily dose of 50 IU/kg, the combination of a daily dose of vitamin D (50 IU/kg) and Dexa, or the bolus dose of vitamin D (60000 IU/kg) with a daily dose of Dexa, respectively. However, the combination of a daily dose of vitamin D with Dexa, or the bolus dose of vitamin D (60000 IU/kg) with a daily dose of Dexa produced significantly reduced levels of NO ($p < 0.05$) by comparison with the Dexa treatment group (Fig. 1E). However, we did not find any significant changes in serum levels of NO between the groups treated with vitamin D at the dose of 50 IU/kg or the dose of 60000 IU/kg.

Levels of blood glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides under different treatment conditions

The animals treated with a daily dose of Dexa presented with hyperglycemia ($p < 0.05$) by comparison with the control group and sensitized group. The increases in hyperglycemia were significantly reduced in the group treated with vitamin D ($p < 0.05$) and in the groups treated with the combination of both drugs (Fig. 2A). However, when comparing the groups that received vitamin D at the dose of 50 IU/kg with the dose of 60000 IU/kg, we did not find any significant differences in hypercholesterolemia, hypertriglyceridemia, or serum levels of LDL-C and HDL-C.

Chronic administration of Dexa induced dyslipidemia characterized by hypercholesterolemia ($p < 0.05$), hypertriglyceridemia ($p < 0.05$), and decreased serum levels of HDL-C ($p < 0.05$), by comparison with the control group and sensitized group. We also observed an increase in the LDL-C ($p < 0.05$) levels in animals treated with glucocorticoid. The vitamin D treatment was effective at preventing hypercholesterolemia ($p < 0.05$) (Fig. 2B), hypertriglyceridemia ($p < 0.05$) (Fig. 2C), LDL-C ($p < 0.05$) (Fig. 2D), and increasing HDL-C ($p < 0.05$) (Fig. 2E) induced by the chronic administration of Dexa.

Effect of the treatments on psychological behavior

Open field test

Chronic administration of Dexa produced a significant reduction in ambulatory behavior (crossing) in the open field test by comparison with the control group and sensitized group ($p < 0.05$). Treatment with vitamin D or the drug combination (vitamin D + Dexa) resulted in significant increases ($p < 0.05$) in ambulation as compared with Dexa-treated group (Fig. 3A). Vitamin D at the dose of 60000 IU/kg more significantly ($p < 0.05$) increased ambulation by comparison with vitamin D at the dose of 50 IU/kg.

Marble burying test

Chronic administration of Dexa produced an increase in the number of marbles buried ($p < 0.05$) compared with the control group and the sensitized group. Treatment with vitamin D or the combination of both drugs resulted in a significant decrease in the number of marbles buried ($p < 0.05$) by comparison with the Dexa-treated group (Fig. 3B). However, we did not find any significant differences in the number of marbles buried when comparing the groups treated with vitamin D at the dose of 50 IU/kg and the dose of 60000 IU/kg.

Tail suspension test

Chronic administration of Dexa produced an increase in the duration of immobility ($p < 0.05$) by comparison with the control group and the sensitized group. Treatment with vitamin D or the combination of both drugs significantly ($p < 0.05$) reduced this increase in duration of immobility ($p < 0.05$) by comparison with the Dexa-treated group (Fig. 3C). Treatment with vitamin D at the dose of 60000 IU/kg more significantly ($p < 0.001$) reduced the

duration of immobility when compared with vitamin D at the dose of 50 IU/kg.

Discussion

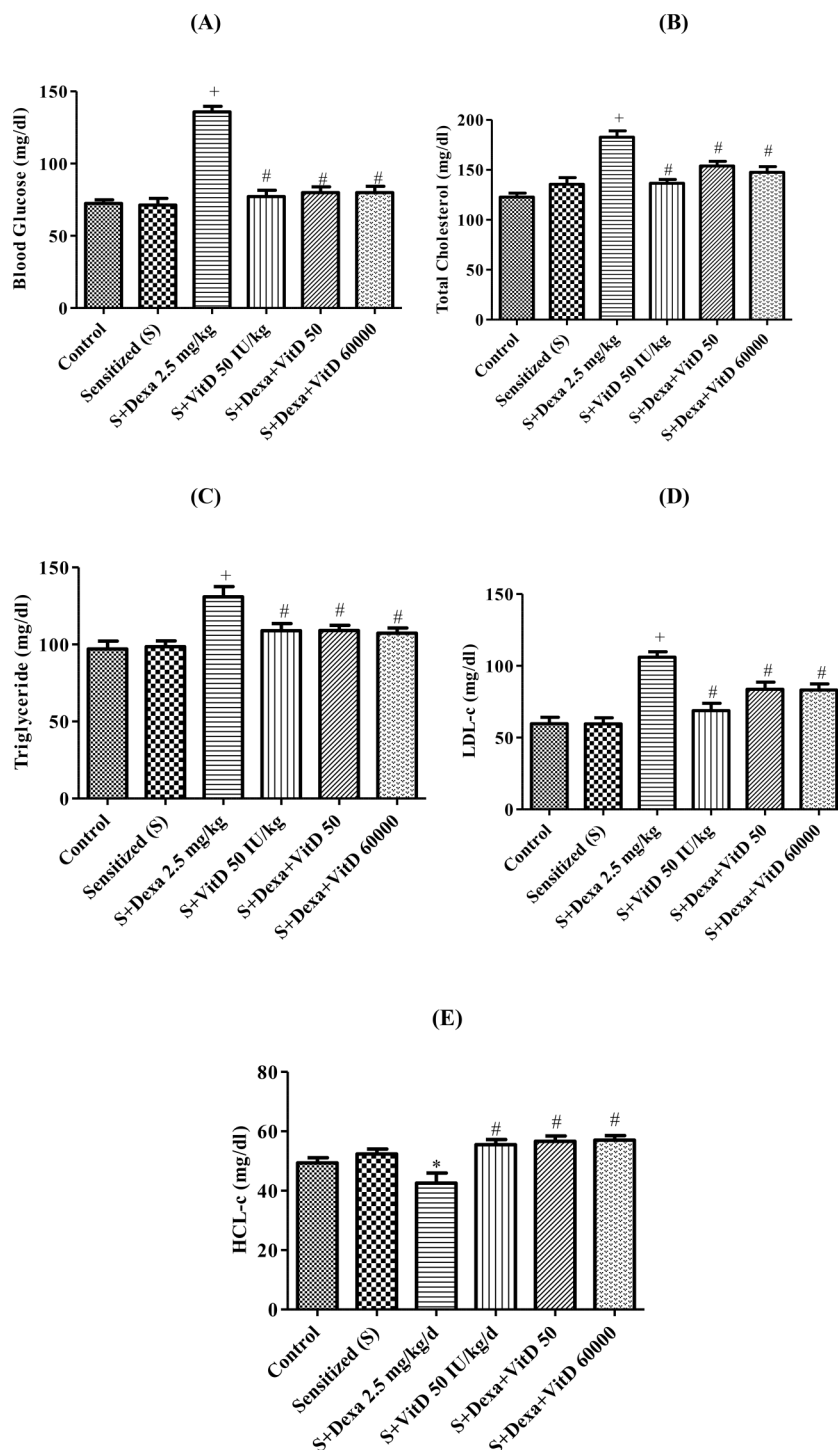
Glucocorticoids (GCs) are the most effective therapy for the treatment of asthma, and currently, the systemic administration of GCs has become first-line of treatment for acute severe asthma, whereas topical (i.e., inhaled) GCs are the first-line agents for the long-term management of all patients with persistent asthma. However, the long-term administration of GCs is associated with multiple adverse effects, including hyperglycemia, hypertension, cardiometabolic abnormalities, adverse psychiatric effects, adrenal insufficiency, weight gain, increased skin fragility, myopathy and cataracts, and mood changes (Chambers and Hawrylowicz 2011). Recently, research has found that vitamin D may have a role in asthma. Recent data have shown that $1\alpha,25(\text{OH})_2\text{D}_3$ has direct effects on activated helper T cells, regulatory T cells, activated B cells, and dendritic cells (Adams and Hewison 2008; Hewison 2010; Cantorna 2011; Jones et al. 2012).

The data presented in this study show that pretreatment with vitamin D or the combination of vitamin D and Dexa in this asthma model decreased the production of inflammatory cells such as eosinophils, neutrophils, lymphocytes, and monocytes, as well as the total cell count. Th2 cells play a key role in the initiation and progression of allergic asthma through the induction of cytokine secretion. In particular, IL-4 and IL-5 lead to eosinophil-rich inflammation in the lungs, and enhanced IgE production and mucus hypersecretion by epithelial goblet cells (Shevach 2002). However, treatment with vitamin D or vitamin D combined with Dexa significantly reduced the levels of IL-5 and IgE in serum and BALF. These results suggest that vitamin D has protective effects in allergic asthma by reducing in the levels of Th2 cytokines, and that it acts synergistically with GCs. Vitamin D is known for its beneficial effects in diseases with strong Th1 responses, perhaps by altering the Th1/Th2 balance in vivo (Lange et al. 2009). Matheu et al. (2003), using a mouse model of allergic asthma, reported that treatment with $1,25(\text{OH})_2\text{D}_3$ reduced airway eosinophilia, whereas Topilski et al. (2004) reported that treatment with $1,25(\text{OH})_2\text{D}_3$ suppressed eosinophilia and lung inflammation scores in a model of allergic asthma induced in either BALB/c or C57BL/6 mice. They also observed that $1,25(\text{OH})_2\text{D}_3$ treatment inhibited chemokine-induced migration of T cells. Wittke et al. (2004) reported that the VDR is believed to be involved in the allergic asthma response in mice, because in their experiments VDR-knockout mice showed mild symptoms of airway inflammation and failed to develop experimental asthma.

Recent findings have implicated NO in the pathogenesis of inflammation in asthma. Excessive production of NO in asthma may contribute to the pathological changes such as airway edema, mucus production, and inflammatory cellular infiltration, particularly during asthma exacerbation (Chang et al. 2004). In our study, vitamin D and the combination of vitamin D and Dexa significantly reduced NO production. Previous reports have shown that $1,25(\text{OH})_2\text{D}_3$ protects against oxidative injuries caused by an NO surge (Chang et al. 2004). Zhou et al. (2008) reported that pretreatment with vitamin D significantly reduced OVA-triggered up-regulation of iNOS and increase in NO levels.

GCs are the most common cause of drug-induced diabetes, which is similar to type 2 diabetes because GCs cause β -cell dysfunction and impair glucose metabolism by increasing insulin resistance in the liver, with increased basal glucose production and impaired glucose utilization in the adipose and skeletal tissues (Clare and Thurby-Hay 2009; van Raalte et al. 2009). In our study, we found that Dexa-induced hyperglycemia is attenuated with vitamin D. Vitamin D is necessary for normal insulin secretion (Chertow et al. 1983; Tanaka et al. 1984) and also reduces insulin resistance, probably through up-regulation of the insulin receptor gene

Fig. 2. (A) Blood glucose, (B) total cholesterol, (C) triglycerides, (D) low-density lipoprotein cholesterol (LDL-c), and (E) high-density lipoprotein cholesterol (HDL-c) from male albino Wistar rats. Values shown are the mean $\pm$ SEM ($n = 6$ rats per group); +, $p < 0.05$ compared with the non-sensitized control group and the sensitized group; #, $p < 0.05$ compared with the dexamethasone-treated group; *, $p < 0.05$ compared with the non-sensitized group. S, ovalbumin sensitized; VitD, vitamin D; Dexa, dexamethasone.

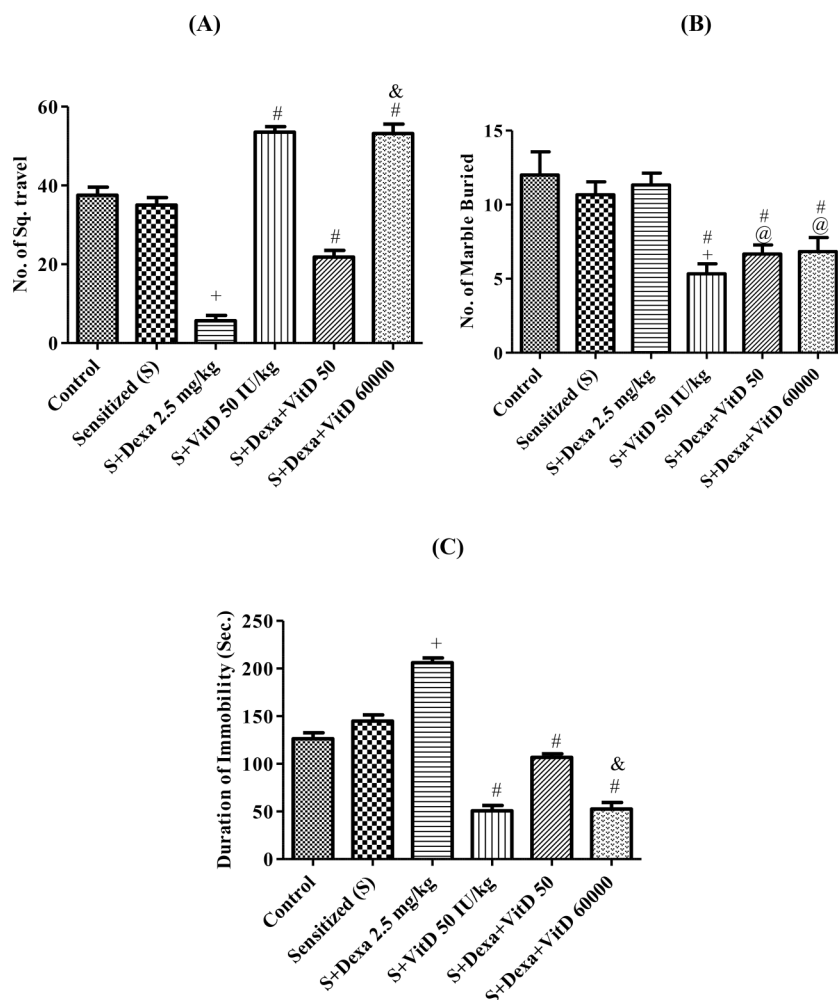


(Maestro et al. 2002). The protective effects from diabetes associated with vitamin D may be due to its anti-inflammatory properties. Vitamin D attenuates the expression of proinflammatory cytokines involved in insulin resistance, such as IL-1, IL-6, and tumor necrosis factor alpha (TNF- α), and also down-regulates nuclear factor kappa B (NF- κ B) activity (Cohen-Lahav et al. 2007). Vitamin D also regulates the nuclear peroxisome proliferator-activated receptor, which has an important role in the insulin

sensitivity (Dunlop et al. 2005). Thus, the combination of Dexa with vitamin D helps to prevent the hyperglycemic side-effects that are induced by Dexa.

GCs also have direct effects on circulating lipids and lipoproteins through increasing serum levels of LDL-C, HDL-C, and triglycerides. These lipid changes can occur within 48 h of initiation (Zimmerman et al. 1984; Ettinger and Hazzard 1988). However, some studies have shown that HDL levels are low in

Fig. 3. Results from the (A) open field test, (B) marble burying test, and (C) tail suspension test on male albino Wistar rats. Values shown are the mean $\pm$ SEM ($n = 6$ rats per group); +, $p < 0.05$ compared with the non-sensitized control group and the sensitized group; @, $p < 0.05$ compared with the non-sensitized control group; #, $p < 0.05$ compared with the dexamethasone-treated group; &, $p < 0.05$ compared with the group treated with vitamin D at the dose of 50 IU/kg. S, ovalbumin sensitized; VitD, vitamin D.



humans receiving corticosteroids, whereas others have reported an increase in HDL after short-term corticosteroid treatment. GCs decrease the concentration of LDL-C receptors on hepatocytes (Rainey et al. 1992), which leads to higher LDL-C levels. GC administration leads to hypertriglyceridemia through the increased production and secretion of very low density (VLD) lipoprotein from the liver (Brindley 1995). Previous studies have examined the relationship between 25(OH) vitamin D and HDL-C, LDL-C, total cholesterol, and triglyceride levels in the plasma (Boon et al. 2007; Hypponen et al. 2008; Reis et al. 2008; Gannage-Yared et al. 2009; Karhapää et al. 2010) and reported an overall positive correlation between 25(OH) vitamin D and HDL-C, and an inverse correlation between 25(OH) vitamin D and LDL-C, total cholesterol, and triglyceride levels. Thus, in our study, Dexa-induced dyslipidemia is attenuated by vitamin D; this effect may be due to the direct and indirect effects of vitamin D on serum levels of lipids.

In this study, the chronic administration of corticosteroids induced depression-like behavior in the tail suspension test and anxiety-like behavior in open field test and marble burying test. The biological mechanisms by which corticosteroids may affect behavior are not fully understood, but evidence suggests that corticosteroids have direct and indirect effects on the central nervous system in animals and in humans (McEwen et al. 1979; Scheff and Cotman 1982; Sapolsky and Pulsinelli 1985; Rothschild et al. 1985; Wolkowitz et al. 1986; Mileusnic et al. 1986; Majewska 1987). Many

of the effects of corticosteroids on the central nervous system are mediated by genomically related events. Corticosteroids alter the transcription of specific genes that code for tyrosine hydroxylase (Van Loon et al. 1977; McEwen et al. 1979; Stene et al. 1980), monoamine oxidase (McEwen et al. 1979; Stene et al. 1980), and dopamine/3-hydroxylase (McEwen et al. 1979), which are enzymes that control the activity of catecholamines and other neurotransmitters. Some data have been produced that suggested GCs act within 24 h to significantly increase the rate of apoptosis in the hippocampus (Yu et al. 2008), probably via inhibition of glucose uptake in neurons (Sapolsky 1996; Mirescu and Gould 2006), leading to decreased hippocampal volume and performance (Lloyd et al. 2004). Calcitriol activates the gene expression of the enzyme tyrosine hydroxylase (Dunlop and Nemeroff 2007), which leads to increased availability of dopamine, noradrenaline, and adrenaline, which are involved in the pathophysiology of mood disorders (Puchacz et al. 1996; Humble 2000; Kalueff et al. 2004; Dunlop and Nemeroff 2007; Drevets and Furey 2010). Kalueff et al. (2004) showed that mice deficient in the VDR gene demonstrate increased anxiety-like behaviour. Several studies have shown that increases in vitamin D levels significantly reduce depression-like behavior (Lansdowne and Provost 1998; Gloth et al. 1999; Shipowick et al. 2009; Vieth et al. 2004; Jorde et al. 2008). In our study, we found that vitamin D and its combination with Dexa significantly reduced anxiety and depression-like behavior, either by involving genomic or nongenomic mechanisms through increasing

the levels of various neurotransmitters that are involved in the pathophysiology of mood disorders.

Previous studies on humans have shown that low daily doses of vitamin D have better therapeutic effects than high bolus doses (Bergman et al. 2013), but in our study we did not find any statistically significant difference in anti-asthmatic activity or the associated side-effects induced with corticosteroids when comparing the low daily dose and the high bolus dose of vitamin D, although there was a difference found in the study of psychiatric side-effects: the bolus dose of vitamin D was more effective than the daily dose in reversing anxiety and depression-like behavior. This difference may be because our study used rats, and may also be associated with a difference in study duration.

In conclusion, the administration of vitamin D in combination with Dexamethasone in this asthma model significantly decreased the number of inflammatory cells, cytokines, and Dexamethasone-associated side-effects. Thus, treatment with vitamin D along with Dexamethasone could be beneficial clinically for enhancing Dexamethasone activity, and help to reduce Dexamethasone-associated side-effects.

Conflict of interest: The authors declare that there are no conflicts of interest associated with this study.

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