Pharmacological basis of the putative therapeutic effect of Topical Vitamin D3 on the experimental model of atopic dermatitis in mice

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Abstract. – OBJECTIVE: The aim of the study was to explore the effect of topical vitamin D3 in atopic dermatitis (AD) induced by ovalbumin (OVA) in contrast with topical betamethasone in mice.

MATERIALS AND METHODS: 35 BALB/c adult male mice, weighing between 25-30 gm were used to induce AD by topically sensitizing the dorsal surface of the skin with the OVA patch. Subsequently, treatments were performed in each group by application of vitamin D3 cream (0.0003%), betamethasone cream (0.1%), or vehicles (QV cream) on the skin.

RESULTS: Remarkably, vitamin D3 had a marked improvement in the skin of OVA-induced AD mice. Additionally, vitamin D3 revealed a considerable diminution in the levels of IgE, IL-5, filaggrin, and epidermal thickness, whereas a significant augmentation in the levels of IL-4 and IL-13 was observed when compared with the control group, and histopathological studies had further confirmed these findings.

CONCLUSIONS: This study essentially highlighted the anti-inflammatory effect of vitamin D3 by effective alteration in the immunological components responsible for AD. Moreover, this pioneer experimental work represents a new paradigm and sheds a light on the importance of vitamin D3 in the implications of AD. A comprehensive creative approach is crucial to concretely establish and further corroborate vitamin D3 for this therapeutic role.

Key Words: Atopic dermatitis, Vitamin D3, OVA-induced mice model for AD, Cytokines.

Introduction

One of the common persistent and often relapsing skin disorders is recognized as Atopic Dermatitis (AD). The basic essence of its etiology is characterized by the intricate interaction between the inherent genetic and immunological components of the patient and certain environmental factors. Basically, AD is exemplified by chronic inflammation, interruption of epithelial barrier, and aberration of the immunological status invariably with escalated levels of serum IgE.

Accumulated evidence revealed that a remarkable skin barrier defect is displayed in the epidermal layer with a basic defect, leading to trans-epidermal water loss; this in turn alters the skin sensitivity. Furthermore, allergen penetration and microbial colonization with imminent inflammation ensue. The resultant skin barrier defect is primarily attributed to certain vital factors like a deficiency in structural proteins, such as filaggrin and keratin, changes in the pH of stratum corneum, diminution of skin ceramide, which is responsible for lipid barrier and water retention. Nevertheless, the most remarkable genetic factor responsible for the disposition to AD seems to be the mutation in the filaggrin gene. Interestingly, 50% of the population studies have revealed filaggrin mutations.

The contemporary acquaintance with the function of vitamin D in the last few decades unlocked a new vista for its extra-skeletal effect. Seemingly, it became quite apparent to portray its vital facets in cell proliferation regulation and their differentiation, in addition to the modulation of the immune system.

This regulatory action of vitamin D is typically recognized as its genomic action. Fascinatingly, vitamin D has been implicated in several stud-
ies\textsuperscript{5}, regarding its contributory effect on maintaining appropriate epidermal barrier which is disrupted by the application of corticosteroids. Furthermore, the mediation of this effect of vitamin D was attributed to its effect on the enhancement of the synthesis of the cornified envelope’s structural protein. In addition, it modifies the ultimate synthesis of the long chain glucosylceramide, which is quite necessary for the formation of lipid barrier\textsuperscript{6}.

Moreover, in many observational studies and meta-analysis\textsuperscript{7-9}, there is a reciprocal and inadvertent relationship between AD and deficiency of vitamin D in children, as well as adults. Besides, the severity of AD has been observed to be negatively correlated with vitamin D levels. Notwithstanding it was categorically proposed that restoration of the transformed Th1 and Th2 cytokines, the upregulation of IL-2, IL-4, IL-6 and IFN-\textgamma in the patients affected with AD can explicitly be successfully reduced by the supplementation of vitamin D\textsuperscript{10}. Conversely, the Th2 cytokines decrease the expression of FLG protein and antimicrobial peptides (AMPs), and these AMPs have an essential role against infections\textsuperscript{11}.

The contemporary therapeutic remedy for AD includes topical corticosteroids and topical calcineurin inhibitors (immunosuppressive agents), nevertheless, their chronic therapy leads to numerous adverse effects. In contrast, newer drug therapy for AD approved by FDA, e.g., crisaborole and dupilumab, demonstrated to be highly expensive in terms of cost effectiveness\textsuperscript{12}.

This study essentially aims at scrutinizing the potential curative effect of vitamin D\textsubscript{3} against the therapeutic impact of corticosteroids in the mice model of AD. Moreover, to offer a concept of IgE, Th2- cytokines (IL-5, IL-4 and IL-13), and filaggrin implication, with the supportive role of histological study to confirm the effectiveness of vitamin D.

Materials and Methods

Chemical and Reagents

\textit{1α, 25-dihydroxy vitamin D\textsubscript{3}}, and chicken ovalbumin egg white (OVA) (Sigma Aldrich CO., Saint Louis, MO, USA) were used. Betamethasone cream (15 g), 0.1% w/w betamethasone valerate, (Betasone\textsuperscript{®}, JULPHAR, UAE). QV\textsuperscript{®} cream (Ego Pharmaceuticals, Austria) was applied. ELISA kits for immunological parameters IgE, IL-4, IL-5, and IL-13 (MyBioSource Inc., USA). All other chemicals are of analytical grade.

Formulations

Vitamin D\textsubscript{3} cream was daily prepared. An appropriate amount of QV\textsuperscript{®} cream (vehicle) was added in geometric dilution to Vitamin D\textsubscript{3} solution in accordance with the protocol observed in Chauhan et al\textsuperscript{13}. Furthermore, to prepare 1 gram of the medicated cream of final concentration, 3 µg/g (0.0003% w/w) were prepared according to the validated technique\textsuperscript{14}.

Animal and Housing

BALB/c adult male mice, weighing between 25-30 gm, were obtained from KFCMR, KAU, Jeddah, Saudi Arabia. The animals were divided in groups of seven and were kept in a stable environment at room temperature of 24°C with a 12-hour natural light/dark cycle. Free access to food and water was provided \textit{ad libitum} during the entire span of the experiment.

Study Design

In this contemporary study, 35 mice were acquired and divided randomly in five groups (n=7) as follows: 1-Normal control group (NC); 2-OVA-induced AD group (OVA); 3-OVA-induced AD + QV vehicle group (OVA+QV); 4-OVA-induced AD + vitamin D\textsubscript{3} cream treated group (VDTC), 5-OVA-induced AD + betamethasone cream treated group (BET).

Allergic Dermatitis Induction in Mice

AD was induced topically in the mice as follows: the dorsal skin of all mice was shaved and sensitized topically with the OVA patch. Then the OVA patch was attached to the shaved dorsal skin using a transparent dressing, then fixed with silk tape. The patches were renewed every other day for 14 days (from day 14 to 27). After the AD induction, the mice in each group were treated according to the protocol: from day 30 to 34, 3 µg/g (0.0003%) of Vitamin D3 cream, betamethasone cream (0.1%), or vehicles (QV cream) were applied directly on the dorsal skin.

Sample Collection

All mice were subjected to anesthesia by diethyl ether and samples of the blood were subsequently obtained from the orbital sinus in the heparinized capillary tubes. Finally, the mice were sacrificed.
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by decapitation\textsuperscript{15}, and the dorsal skin was dissected out and divided into two parts. One part was frozen at -80°C for the measurement of immunological parameters, and the other part was stored in 10% of buffered formalin for the histopathological studies.

\textbf{Assessment of AD Appearance and its Severity}

It needs to be emphasized that the development of AD was confirmed by the Three-Item Severity Score (TIS), which is the scoring system to determine the severity of AD. Its significance well corresponds with the scoring for the atopic dermatitis assessment\textsuperscript{16}. The common parameters for the TIS scoring are erythema (redness), edema (swelling), and excoriations (scratched abrasion). Each sign is scored as: 0 = clear; 1 = mild; 2 = moderate; 3 = severe\textsuperscript{16,17}.

\textbf{Serum Samples}

The samples of blood obtained were duly centrifuged at 3,000 rpm for a period of 20 minutes. Consequently, the resultant serum was collected in Eppendorf tubes and then stored at -80°C until the assays were accomplished.

\textbf{Skin Tissue Homogenate Preparation}

To accomplish this task, 10 mg of tissues were minced to small pieces and homogenized in 100 µl of PBS. Then, the subsequent suspension obtained was exposed to ultra-sonification for the purpose of breaking the cell membrane. This was followed by centrifugation of the homogenate at 5,000 rpm for 15 minutes duration. Finally, the supernatants were carefully obtained and stored at -80°C till the assays were done.

\textbf{Biochemical Estimations}

The levels of IgE, IL-5, IL-4, IL-13, and filaggrin in serum and tissue homogenate were prepared by using ELISA kits manufactured by MyBioSource (San Diego, CA, USA) following the manufacturer’s recommended procedures. It needs to be emphasized that all the kits acquired belong to the sandwich type, except for IgE ELISA kit, which is designated as a competitive type. Moreover, the absorbance was measured at $\lambda = 450$ nm, by using the BIO-TEK ELx 800 plate reader (VT, USA).

\textbf{Histopathological Examination}

The harvested skin was spread on a plastic cassette fixed in 10% buffered formalin. This was further processed by an automatic paraffin processing machine to prepare the blocks. Subsequently, for general structure and morphometric studies, the blocks were sectioned into 5 µm thick slices and mounted on slides. This was followed by Hematoxylin and Eosin (H&E) staining. Then, the slides were photographed using an Olympus Microscope BX-51 at the magnification of 4X with a digital camera connected to the computer, in accordance with the established method\textsuperscript{18}.

\textbf{Statistical Analysis}

The data analysis was accomplished by the utilization of the Social Package of Social Science (SPSS), version 23 (IBM Corp., Armonk, NY, USA). Typically, the data were expressed as mean ± SEM. Moreover, we used one-way analysis of variance (ANOVA) to examine statistical comparisons between more than two independent groups, followed by the Least significance difference (LSD) test. However, we used an independent sample \textit{t}-test to compare the two groups. Finally, consideration of the significance of differences was expressed as \( p < 0.05 \).

\textbf{Results}

\textbf{Effect of Daily Topical Administration (TA) of Vitamin D3 Cream (3 µg/g) over Betamethasone Cream for Five Days on OVA-Induced AD Mice}

The VDTC and BET groups showed a marked improvement in the skin appearance compared to the OVA group. Vitamin D3 treatment ameliorated AD in the VDTC group and scored 0 (clear) for erythema and edema, while it scored 1 (mild) for excoriations (Figure 1).

\textbf{Effect of Daily TA of Vitamin D3 Cream (3 µg/g) over Betamethasone Cream for Five Days on Serum and Skin Homogenate IgE Levels (ng/ml) in OVA-Induced AD in Mice}

The result in Figure 2 illustrates a significant increase (\( p < 0.05 \)) in the serum and skin homogenate IgE levels for the OVA and vehicle OVA+QV group in comparison with the control NC group. However, a significant reduction (\( p < 0.05 \)) was shown in the serum and skin homogenate IgE levels for the vitamin D\textsubscript{3} treated VDTC and betamethasone treated BET group compared to the OVA group (Figure 2).
Effect of Daily TA of Vitamin D3 Cream (3 µg/g) vs. Betamethasone Cream for Five Days on Serum and Skin Homogenate IL-5 Levels (pg/ml) in OVA-Induced AD in Mice

As shown in Figure 3, there was a significant increase (p < 0.05) in the serum and skin homogenate IL-5 levels for the OVA and vehicle OVA+QV group relative to the control NC group. However, a significant decrease (p < 0.05) in the serum and skin homogenate IL-5 levels was observed for the vitamin D₃ cream treated VDTC and betamethasone cream treated BET group compared to the OVA group. Moreover, the VDTC group showed a significant increase (p < 0.05) in the serum and skin homogenate IL-5 levels compared to the BET group (Figure 3).

Effect of Daily TA of Vitamin D3 Cream (3 µg/g) vs. Betamethasone Cream for Five Days on Serum and Skin Homogenate IL-4 Levels (pg/ml) in OVA-Induced AD in Mice

The data in Figure 4 display a significant decrease (p < 0.05) in the serum and skin homogenate IL-4 levels relative to the control NC group for the OVA and vehicle OVA+QV group. Moreover, VDTC and BET group demonstrated
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A significant increase ($p < 0.05$) in the serum and skin homogenate IL-4 levels in comparison to the OVA group (Figure 4).

Effect of Daily TA of Vitamin D3 Cream (3 µg/g) vs. Betamethasone Cream for Five Days on Serum and Skin Homogenate IL-13 Levels (pg/ml) in OVA-Induced AD in Mice

A significant augmentation ($p < 0.05$) was demonstrated in the serum and skin homogenate IL-13 levels for the Vitamin D3 cream treated VDTC and betamethasone cream treated BET group compared to the OVA group. Additionally, the VDTC group showed a significant decrease ($p < 0.05$) in the serum and skin homogenate IL-13 levels vs. the BET group (Figure 5).

Table I. Effect of vitamin TA of vitamin D3 cream over betamethasone cream on the OVA-induced increased skin homogenate filaggrin (ng/ml).

<table>
<thead>
<tr>
<th>Groups (n = 7)</th>
<th>Skin homogenate filaggrin (ng/ml) mean ± SEM</th>
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<tbody>
<tr>
<td>Normal control group (NC)</td>
<td>2.50±0.16</td>
</tr>
<tr>
<td>OVA-induced AD group (OVA)</td>
<td>8.15±0.27</td>
</tr>
<tr>
<td>OVA-induced AD + QV vehicle group (OVA+QV)</td>
<td>5.92±0.37†††</td>
</tr>
<tr>
<td>OVA-induced AD + vitamin D3 cream treated group (VDTC)</td>
<td>2.52±0.16∗∗∗</td>
</tr>
<tr>
<td>OVA-induced AD + betamethasone cream treated group (BET)</td>
<td>3.21±0.24∗∗∗</td>
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∗Significant $p$-value $< 0.05$ vs. NC group, †Significant $p$-value $< 0.05$ vs. OVA group, ‡Significant $p$-value $< 0.05$ vs. OVA+QV group.

The data provided in Table I revealed quite significant augmentation ($p < 0.05$) in the skin homogenate filaggrin level induced for the OVA and vehicle OVA+QV group relative to the control NC group. On the other hand, VDTC and BET group, a significant decrease ($p < 0.05$) in the level of skin homogenate filaggrin was registered in comparison to the OVA group.

Effect of TA of Vitamin D3 Cream (3 µg/g) and Betamethasone Cream for Five Days in OVA-Induced AD in Sections of the Mice Skin

The NC control group has a typical appearance (double head arrow) and thickness of both

![Figure 3](image-url)

Figure 3. Effect of daily TA of vitamin D3 cream (3 µg/g) vs. betamethasone cream for five days on serum and skin homogenate IL-5 levels (pg/ml) in OVA-induced AD in mice. Significant $p$-value $< 0.05$ vs. (NC) group, ‡Significant $p$-value $< 0.05$ vs. (OVA) group, ‡‡Significant $p$-value $< 0.05$ vs. (OVA+QV) group, ‡‡‡Significant $p$-value $< 0.05$ vs. (BET) group.
The fundamental hallmark of this condition is its association with an increased serum-IgE level. It needs to be emphasized that the characteristic features of OVA-induced AD are: epidermal thickening, immune-dominated responses with upregulated expression of Th2 cytokines (IL-5, IL-4, and IL-13), elevated IgE level and infiltration of inflammatory cells. To reiterate that, this model was preferred for the current study because the OVA-induced AD model displays many human AD features and seemingly BALB/c mice are more suitable for immunological studies.

In the present study, regarding the AD appearance and skin condition, the group treated with vitamin D3 cream demonstrated a marked healing enhancement in skin condition as compared to OVA group. On the other hand, before dissection, the OVA group appeared to have recovered, but they still displayed moderate AD symptoms according to the TIS score. The OVA group mice underwent the subacute or interphase stage of AD (4-5 weeks) with moderate symptoms compared to the NC group (Figure 1). Concerning the effect of vehicles on AD, the skin of the vehicle group (OVA+QV) showed a marked improvement in skin appearance compared to the OVA group. At the same time, they had AD symptoms compared to the NC group. This pertinent finding agreed

Discussion

A noteworthy and realistic fact designates that atopic dermatitis is a widespread and recurrent inflammatory skin disorder. Furthermore, it is exemplified by the key symptoms of severe pruritis, dehydrated dry skin, swelling and eczematous lesions. The fundamental hallmark of this condition is its association with an increased serum-IgE level. It needs to be emphasized that the characteristic features of OVA-induced AD are: epidermal thickening, immune-dominated responses with upregulated expression of Th2 cytokines (IL-5, IL-4, and IL-13), elevated IgE level and infiltration of inflammatory cells. To reiterate that, this model was preferred for the current study because the OVA-induced AD model displays many human AD features and seemingly BALB/c mice are more suitable for immunological studies.

In the present study, regarding the AD appearance and skin condition, the group treated with vitamin D3 cream demonstrated a marked healing enhancement in skin condition as compared to OVA group. On the other hand, before dissection, the OVA group appeared to have recovered, but they still displayed moderate AD symptoms according to the TIS score. The OVA group mice underwent the subacute or interphase stage of AD (4-5 weeks) with moderate symptoms compared to the NC group (Figure 1). Concerning the effect of vehicles on AD, the skin of the vehicle group (OVA+QV) showed a marked improvement in skin appearance compared to the OVA group. At the same time, they had AD symptoms compared to the NC group. This pertinent finding agreed

Figure 4. Effect of daily TA of vitamin D3 cream (3 µg/g) vs. betamethasone cream for five days on serum and skin homogenate IL-4 levels (pg/ml) in OVA-induced AD in mice. *Significant p-value < 0.05 vs. (NC) group, **Significant p-value < 0.05 vs. (OVA) group, $$$Significant p-value < 0.05 vs. (OVA+QV) group.
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vitamin D deficiency was reported in mice with a higher IgE level. Their data suggested that vitamin D potentially controls the IgE response through direct and indirect regulation. The direct effect of vitamin D was demonstrated, where the B cells significantly produce IL-10 following OVA immunization, which in turn suppresses the IgE synthesis. In addition, the supportive role of vitamin D was also identified as a potent anti-inflammatory agent due to its action on the immune system’s innate and adaptive cells.

Apparently, in the adaptive immune system, vitamin D3 treatment decreases the activation, differentiation, and proliferation of B cells leading to reduced immunoglobulin (Ig) production from B cells. This control of B cell activation by vitamin D plays a significant role in inflammatory diseases. These results strongly indicate that vitamin D has a regulatory role in IgE production.

We wish to enlighten that, in the contemporary study the effect of vitamin D3 on the inflammatory cytokines IL-5, IL-4, and IL-13 levels was also investigated (Figure 3, 4 and 5). The data obtained demonstrated that the OVA and OVA+QV groups showed a significant augmentation in the serum and skin homogenate IL-5 levels (Figure 3), while vitamin D deficiency was reported in mice with a higher IgE level. Their data suggested that vitamin D potentially controls the IgE response through direct and indirect regulation. The direct effect of vitamin D was demonstrated, where the B cells significantly produce IL-10 following OVA immunization, which in turn suppresses the IgE synthesis. In addition, the supportive role of vitamin D was also identified as a potent anti-inflammatory agent due to its action on the immune system’s innate and adaptive cells.

Moreover, in the present study, the OVA and OVA+QV groups demonstrated a significant augmentation in the serum and skin homogenate IgE levels in comparison with the NC group (Figure 2). On the other hand, a significant decrease in the serum and skin homogenate IgE levels was observed for the VDTC, and BET groups compared to the OVA group. These findings agree with a previous study, which demonstrated that vitamin D treatment significantly suppresses the IgE levels. The authors found that vitamin D regulates the serum-IgE level in vivo; besides,
significant diminutions in the IL-4 and IL-13 levels (Figure 4 and 5) were observed as compared to the NC group. These results are consistent with other studies that dealt with the OVA-induced AD mice model, where an increase in the IL-5, IL-4, and IL-13 levels were reported.

We wish to emphasize that in our study a significant upsurge in the IgE and IL-5 levels in serum and skin homogenate (Figure 3) was registered, concomitantly with a significant reduction in the serum and skin homogenate IL-4 and IL-13 levels (Figure 4 and 5), and the time-dependent progression suggested that the mice might have been in the interphase or subacute phase of AD.

The underlying details of VDTC and BET groups demonstrated a significant decrease in the serum and skin homogenate IL-5 levels (Figure 3), and a significant increase in the serum and skin homogenate IL-4 and IL-13 levels compared to the OVA group (Figure 4 and 5). Concurrently, a non-significant difference was also observed in the serum and skin homogenate IL-4 levels, only in the VDTC group vs. BET group comparison (Figure 4). Intriguingly, this is in accordance with a recent study by Szymczak and Pawliczak, affirming that treatment with the active form of vitamin D₃ (calcitriol) inhibits the secretion of pro-inflammatory cells Th1, such as IL-2, INF-γ, and TNF-α, and restores the balance between Th1 and Th2 by increasing the anti-inflammatory effect via promoting the production of T helper Type 2 induced cytokines, such as IL-3, IL-4, IL-5 and IL-10.

Another longstanding focus of interest displayed in this study comprises vital parameters and filaggrin level, which is a protein component of epithelial cells of the skin; interestingly, the skin homogenate filaggrin level was significantly increased in the OVA and OVA+QV groups, compared to the NC group (Table I). These findings are in conformity with previous studies that have documented that the filaggrin levels are reduced in the AD. It has been reported in other studies that filaggrin expression is modulated by the atopic inflammatory responses, specifically Th2 cytokines like IL-4 and IL-13, which modify the expression of filaggrin when differentiation of keratinocyte occurs during the acute phase.

Figure 6. Effect of daily TA of vitamin D3 cream (3 µg/g) vs. betamethasone cream for five days in OVA-induced AD in sections of the mice skin. (× 100 for the first row and × 400 for the second row). NC = normal control, OVA = AD induced by OVA, OVA-induced AD + QV vehicle group VDTC = AD induced by OVA +treated group of vitamin D3 cream, BET = AD induced by OVA+ treated with betamethasone.
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of AD. Moreover, in the presence of the cytokines like IL-4 and IL-13, the expression of filaggrin is substantially decreased.

In contrast, the absence of Th2 cytokines and the presence of IFN-γ, which is a Th1 cytokine control, increase the filaggrin expression. Unequivocally, these results have a positive correlation with the results of our present study and further confirm that the mice were in the subacute phase of AD.

Notably, with the intention of developing targeted base drug therapy, several studies have demonstrated that vitamin D can modulate the proliferation and differentiation of keratinocytes. Interestingly, vitamin D is used to normalize the hyperproliferation present in psoriasis skin, and many studies observed that vitamin D is essential for the generation and maintenance of the skin barrier. In an experimental study by Hong et al., it was shown that filaggrin was higher in the histological levels of mice treated with narrowband UVB phototherapy. This study further extended the approach to substantiate that the vitamin D3 treated groups (Table I) showed decreased filaggrin levels when compared to the OVA control. Furthermore, the histological study (Figure 6) illustrated a significant increase in the epidermal thickness in the OVA and OVA+QV groups compared to the NC group. This has been confirmed in a recent study by Kim et al., demonstrating that the epidermal thickness is increased in mice presenting OVA-induced AD.

The VDTC and BET groups in this study (Figure 6) resulted in a significant decrease in the epidermal thickness compared to the OVA group. These findings agree with the Hong et al. study, which demonstrated that the topical administration of the active form vitamin D3 showed an improvement in the stratum corneum integrity and barrier recovery. In contrast, the vitamin D3 analogs have shown interruption in the permeability barrier of the epidermis by virtue of producing the proliferation of epidermal cells and inducing premature keratinization with no morphological signs of inflammation.

Realistically, AD upsets the routine life of the patients, and its economic burden is quite high. Additionally, the contemporary well-acquainted corticosteroid therapy shows several unacceptable adverse effects. In contrast, vitamin D is quite safe and affordable. Seemingly, it may serve as a novel anti-inflammatory agent, as evident in our present study which revealed almost equivalent results to corticosteroids. It needs to be emphasized that more extensive studies seem to be a pre-requisite requirement for topical vitamin D3 to assess its safety and pharmaceutical stability.

Conclusions

The present study effectively demonstrated the target-based anti-inflammatory effect of vitamin D3 by effective and favorable alteration in the immunological components responsible for AD. Moreover, this pioneer experimental work represents a new paradigm and sheds a light on the importance of vitamin D3 in the treatment of AD. Hence, vitamin D3 may provide a promising future agent for AD treatment.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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None.

Ethics Approval

This experimental study was performed at King Fahd Center for Medical Research (KFCMR), King Abdulaziz University (KAU), Jeddah, Saudi Arabia, and the animal study protocol was approved by the KAU research ethics committee (Approval number: 306-18).

Authors’ Contributions

NSA: formal analysis, experimental work, investigation, MAASA: Conceptualization, data curation, supervision, HMK: visualization, original draft preparation, ASA: methodology, LMK: writing–review and editing, validation, and software. “All authors have read and agreed to the published version of the manuscript.

References