



Expression of Vitamin D Receptor Gene (VDR) and Associated Cytokine Response in Arabian Horses with Chronic Inflammatory Airway Disease



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Abstract

INFLAMMATORY airway disease (IAD) represents inflammatory conditions of the lower respiratory tract. IAD occurs in athletic horses and is a common cause of training interruption and impaired performance. The aim of this investigation was to evaluate the relationship between the Vitamin D Receptor (VDR) gene and cytokines in horses with chronic IAD. Ten horses with IAD and five clinically healthy horses were studied. Serum levels of tumor necrosis alpha (TNF- α) and interleukin-1 β (IL-1 β) were measured, and real-time PCR was used for relative quantification of the mRNA level of the VDR gene in horse blood samples. Clinically, horses under investigation suffered from IAD showing mucopurulent nasal discharge, increased respiratory rate, chronic cough, and abnormal lung and tracheal sounds. A significant increase in IL-1 β ($p < 0.01$) and TNF ($p < 0.01$) levels was recorded in horses with chronic IAD compared to those in clinically healthy horses. However, VDR gene expression was significantly decreased ($p < 0.001$) in horses with chronic inflammatory airway disease compared to that in the controls. There was a positive correlation between IL-1 β and TNF- α levels ($r =$, $p = 0.001$). However, a negative correlation was observed between VDR gene expression and IL-1 β and TNF- α levels. In conclusion, the results of the present study indicate that VDR gene has a major role in the occurrence of IAD in horses. Further studies on the role of vitamin D as a preventive therapy for IAD are required in clinical practice.

Keywords: Respiratory diseases, Equine, IL-1 β , TNF- α , Vitamin D.

Introduction

Respiratory diseases in horses are one of the most common leading problems that require veterinary attention, where early precise diagnosis allows appropriate treatment and satisfies animal's owner [1]. According to the National Equine Health Surveys in the UK, respiratory diseases ranked as the fourth among the diseases affecting leisure horses [2]. Inflammatory airway disease (IAD) affects horses of any age and is prevalent in young

racehorses [3]. Clinically, chronic intermittent cough, increased mucous airway secretion, and exercise intolerance are the main signs [4].

Cytokines interfere with inflammatory disorders such as asthma by triggering, activating, and affecting on the survival rate of inflammatory cells [5]. Proinflammatory cytokines such as tumor necrosis factor (TNF- α)- α and interleukin-1 β (IL-1 β) play a specific role in regulating inflammatory

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responses of the airway [6]. Interleukin-1 β is the primary secreted form of IL-1 and was initially identified as a lymphocyte activation agent that promotes B-cell activation. This cytokine is mainly produced by activated macrophages, dendritic cells, and monocytes and mediates the inflammatory response through various cell activities such as cell proliferation, differentiation, and apoptosis [7]. IL-1 β and TNF- α has been found to play a crucial role in with IAD [8, 9] and recurrent airway obstruction [10].

TNF- α is the most frequently investigated multidirectional cytokine in the TNF family. Under pathological conditions, high levels of TNF- α lead to the occurrence of characteristic inflammatory reactions in many diseases [5]. TNF- α is a pro-inflammatory cytokine [11, 12] that plays a major role in airway inflammation and remodeling in asthma [13]. TNF- α plays an important role in several inflammatory lung diseases as acute respiratory syndrome, asthma, and chronic obstructive pulmonary disease (COPD) [14].

Vitamin D plays a major task in growth, cell differentiation of multiple cells and calcium homeostasis [15]. It activates the cells responsible for both innate immunity and adaptive, with gene expression of the vitamin D receptor (VDR) in response to 1,25-dehydroxycholecalciferol [16]. Vitamin D3, the active form of Vitamin D, not only controls calcium and bone metabolism but also plays a role in immune modulation in monocytes, macrophages, and activated lymphocytes mediated via their receptor binding (VDR) [17]. VDR is present in many immune cells, such as macrophages, dendritic cells, and T and B lymphocytes [18] and plays a complex role in the host immune response after stimulation [19].

To the best of our knowledge, studies on the role of Vitamin D in horses with respiratory diseases are limited. Therefore, the objective of this study was to delineate the association between VDR gene expression and associated cytokines in inflammatory airway disease (IAD) in Arabian horses.

Material and Methods

This study was approved by the Mansoura University Animal Care and Use Committee (MUACUC-Vet:212-2020). A total number of fifteen horses were enrolled in the current study, of which ten horses exhibited signs of chronic IAD and five horses were randomly selected to be clinically healthy. The horses were 1-10 years of age and 230-400 kg of weight. The horses were domestic breed and 1-10 years of age. This study was conducted from December 2017 to November 2018 in the

governorate of Dakahlia, Egypt. The feeding regimen for all horses under study was a balanced ration containing chopped wheat straw, 2.0–5.0 kg of mixed bran and ground corn fortified with trace elements and mineral mixture.

Comprehensive data were collected regarding complete case history, clinical findings, and medical records.

Clinical presentation

A detailed clinical examination of the horses was performed as previously described (20). This examination aimed to reveal evidence of a respiratory disorder (abnormalities in the rate and depth of breathing, chronic cough, nasal discharge, abnormal lung sounds, and poor performance). These symptoms persisted for more than a month. Serum cytokines, including IL-1 β and TNF- α , were measured to ensure a chronic inflammatory response.

Blood samples

A pair of blood samples (5 ml each) were drawn by puncturing the jugular vein of each horse under study. The first sample was collected into anticoagulant containing tube (EDTA) for the detection of the mRNA level of the VDR gene. The second was drawn in a plain tube to separate the serum, which was kept at -80 °C till analysis.

Cytokine measurement

The serum levels of IL-1 β and TNF- α were quantified using equine ELISA kits (CUSABIO, USA). Both tests were performed in two versions and the optical density was determined using the standard chromatography method.

Extraction of RNA and reverse transcription

Whole blood samples were used for extraction of total RNA using the QIAamp® RNA Blood Mini Kit (QIAGEN, Germany) according to the instructions of the manufacturer. Quantitated purity was measured using a nanoparticle spectrophotometer (UV Vis Spectrophotometer Q5000, Thermofischer, USA). cDNA was synthesized from each sample following the manufacturer's protocol (HiSenScript TM RH [-] cDNA synthesis kit (iNtRON Biotechnology, Korea). A total of 20 μ l of reaction mixture was prepared (a total RNA of up to 5 μ l and 10 μ l of 2x RT Reaction). The solution, 1 μ l of the enzyme mixture solution and DNase / RNase-free water up to 20 μ l; the final reaction mixture was put into a thermal cycle and the following program was carried out; reverse transcription at 45 °C for 1 hour, then RTase inhibition at 85 °C for 10 minutes. Finally, cDNA samples were stored at -20 °C.

Primer design

Primers were designed to express the vitamin D receptor gene through alignment of the following sequences of the vitamin D receptor in several species of equine (*Equus caballus* XM_005611070.3, *Equus asinus* XM_014850058.1, *Equus przewalskii* XM_008513615.1). The PCR product was 514 bp (Table 1).

Real-time PCR

The mRNA level of the VDR gene in horse blood samples was quantified using real-time PCR with a Master Mix (SYBR Green with low ROX, Enzynomics, Korea). β -actin was used as the housekeeping gene with the following primer pair sequence (R: CATGGGTGGAATCATACTGAAA, F: GGAGTAAACGGATTTGGCC) (accession number XM_014834961).

Twenty μ l of reaction mixture was made of 10 μ l TOPreal™ Qpcr 2x PreMIX, 1 μ l of cDNA template, and 1 μ l of 10 μ l for each VDR forward and reverse primer (*Equus caballus*) was used (F: ATCCTGACAGATGAGGAGGTG, R: GAGACAAGGCAGGATCTGA) No. Inlet NM_001163959.1) surrounding exons 3 and 4 from the coding region of the VDR, and 7 μ l of sterile ultra-pure DNase-free water was added to bring the total volume up to 20 μ l. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 59.2°C for 15 s, and elongation at 72°C for 30 s. At the end of the amplification phase, melt curve analysis was performed to confirm the specificity of the PCR item. Relative evaluation of mRNA expression was performed using the 2- $\Delta\Delta$ Ct technique [21]. Quantitative real-time PCR was performed to evaluate the expression profile of the VDR gene.

Statistical analysis

Data analysis was performed with commercial software (SPSS for windows V.20, SPSS, Chicago, USA). The data were checked for normal distribution patterns using the D'Agostino-Pearson test. The data were found to be normally distributed; consequently, the means and standard deviation were calculated for each parameter. Data were handled with an independent sample t-test to determine which groups were statistically different. The correlation between the VDR expression pattern and the levels of the selected cytokines was determined using Pearson's correlation. The correlation between the coefficient and p-value was recorded. For the given statistics, results were considered significant at $p < 0.05$.

Results

Clinically, horses under investigation had various clinical signs, including mucopurulent nasal discharge, increased respiratory rate, chronic cough,

abnormal lung sounds, and abnormal tracheal sounds (Table 2).

There was a significant increase in IL-1 β ($P < 0.01$) and TNF ($P < 0.01$) levels in horses with chronic inflammatory airway disease compared to those in clinically healthy horses (Figure 1). However, VDR gene expression was significantly decreased ($p < 0.001$) in horses with chronic inflammatory airway disease compared to that in the controls (Fig. 2).

IL-1 β showed positive correlation with TNF- α levels ($r =$, $p = 0.001$) (Figure 3). However, VDR gene expression showed a negative correlation with IL-1 β and TNF- α levels. (Fig. 4,5).

Discussion

Inflammatory airway disease (IAD) is a common cause of morbidity and mortality in horses, particularly in young horses. IAD receive special attention from owners and veterinarians [22]. The clinical signs associated with IAD are usually non-specific and can be confused with other equine respiratory diseases. Therefore, differential diagnosis is often based on a combination of clinical history, physical signs, and ancillary diagnostic tests [23]. The present investigation evaluated the expression pattern of VDR in horses with chronic inflammatory airway diseases. Based on the patient's history, clinical investigation, and biochemical analyses, lower inflammatory airway disease was diagnosed.

The horses under investigation exhibited various clinical signs of IAD, including exercise intolerance, chronic cough, nasal discharge, and abnormal lung sounds. These findings can be attributed to airway inflammation, stenosis, and the presence of discharges in the inflamed airway. This result is in accordance with the findings of previous reports [24-26].

In the present study, the horses had chronic lower respiratory tract diseases, which could be attributed to misdiagnosis, neglected acute cases, sub-clinical forms of IAD, and shortage of treatment protocols. These findings are in agreement with those reported by Hodgson and Hodgson [27]. The age range of horses in our study was 1-10 years, which is similar to the findings of a previous report by McGorum, et al. [22] who reported that IAD affects horses of any age. Furthermore, the winter predisposition of the affected horses is in accordance with the findings of Rush and Mair [28] and Couëttil, et al. [29] who reported that the seasonal incidence of IAD is associated with environmental or allergic causes.

Since serum cytokine levels help to orchestrate airway inflammation, the diagnosis of chronic inflammatory airway disease in the present study depended mainly on cytokine levels in the serum of

the tested animals. Two proinflammatory cytokines, IL-1 β and TNF- α , were used for this purpose. In the present study, IL-1 β and TNF- α levels were significantly increased in horses with chronic IAD compared to control horses. Interestingly, there was also a positive correlation between IL-1 β and TNF- α . This finding is similar to those found in horses with IAD [8, 9] and recurrent airway obstruction [10]. Immune signals and inflammation incorporating IL-1 β and TNF- α have been found to be important activators of C-X-C chemokines, and their receptors are present on leukocytes and structural cells [30]. In horses with heaves, there was an upregulation of IL-1 β and TNF- α in pulmonary macrophages, with a strong correlation between the expression of IL-1 β and neutrophil percentage [9]. IL-1 β mRNA expression has also been found to be higher in passively sensitized horses than in control [31]. In asthma, IL-1 β potentiates the expression of chemokines, cytokines, complement system proteins, and immune receptors that contribute to inflammation and increase bronchial smooth muscle response [32]. However, in another study, TNF- α was found to be abundant in the airways of asthmatics and to play a crucial role in asthmatic bronchial hyperresponsiveness and airway remodeling [33]. In human patients, IL-1 β has been reported to play a major role in asthma in humans [34, 35]. Moreover, in experimental asthma, IL-1 β was identified as one of the main molecules involved in the induction of an altered airway response in both human and animal studies, according to his direct work on ASM [36]. Rajizadeh, et al. [37] found that in asthmatic rats, there was an increase in IL-1 β levels in the serum and lungs. Sapey, et al. [38] found that the level of IL-1 β in the serum of COPD patients was significantly higher than that in healthy controls.

El-Shimy, et al. [39] and Rajizadeh, et al. [37] recorded a correlation between serum TNF- α levels and disease severity in patients with COPD. TNF- α is also considered a reliable biomarker of the systemic inflammatory response in COPD. This finding is supported by that of Mir, et al. [40] reported that serum TNF levels were higher in patients compared to the controls. Rajizadeh, et al. [37] found that TNF- α levels increased in the serum, and its expression was also increased in the lungs of asthmatic rats. In humans, Kubysheva, et al. [41] reported that the concentrations of TNF- α in patients with COPD, Asthma-COPD (ACO), and bronchial asthma (BA) were higher than those in healthy nonsmokers.

In fact, information regarding the expression of VDR in equine respiratory diseases is scarce. Previous reports have shown that Active form of vitamin D is a positive regulatory factor for innate

and adaptive immune responses, which can regulate many immune cells [42, 43]. Interestingly, 25-(OH) D can also affect lung function by mediating macrophage activity [44]. In asthma, vitamin D inhibits smooth muscle hypertrophy, thus affecting airway remodeling and lung function [45].

In this investigation, we observed a substantial decrease in the expression of the VDR gene in horses with chronic IAD. This finding proposes that lack of Vitamin D receptors is a leading factor for the inefficient immunological response in horses affected with IAD. Similarly, Chen and Xu [46] showed that vitamin D receptor mRNA expression levels in patients with bronchial asthma were significantly decreased than those in the control group ($P < 0.01$). Moreover, the same authors proved that VDR expression in the peripheral blood was decreased in children with asthma. Agrawal, et al. [47] found that the expression of VDR in the lungs of ovalbumin (OVA)-sensitized and challenged mice decreased under inflammatory conditions, which supports recent findings [48]. Such investigations indicate the anti-inflammatory and immunomodulatory functions of Vitamin D in several tissues. The role of VDR in cell differentiation, proliferation, and immune modulation has been clarified [49], as VDR is found in most of cells in the immune system, especially in macrophages and dendritic cells [50, 51].

The results showed that the level of expression was negatively correlated with the levels of TNF- α and IL-1 β . VDR expression in alveolar macrophages of foals and adult horses indicates that its expression is age related [52]. In horses infected with *Rhodococcus equi*, there was a significant decrease in VDR expression, with a significant increase in TNF and IL-1 β in alveolar macrophages [53]. However, other effects could upregulate VDR protein expression in six ponies [54]. These results were also consistent with those of Chen and Xu [46] in humans, who found that TNF- α was negatively correlated with the expression of the VDR gene ($P < 0.05$) but positively correlated with the levels of pro-inflammatory factors. In humans, a negative correlation has been reported between serum 25(OH)D and TNF- α concentrations in healthy women [55]. In patients with COVID-19, TNF and IL-6 levels are inversely correlated with the levels of vitamin D [56] and Vitamin D with IL-1 β [57]. In a mouse model of rheumatic arthritis, there was a negative correlation between VDR signalling and TNF- α concentration [58]. Cantorna, et al. [59] stated that Vitamin D is an important regulator of the immune system and is consequently correlated with immune diseases.

In conclusion, the results of the present study indicate that VDR plays an important role in the

occurrence of IAD in horses. Further studies on the role of vitamin D as a preventive agent for IAD are required in clinical practice.

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Conflicts of Interest

The authors declare no conflict of interest.

Author`s contributions

Authors contributed equally to this work.

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Data Availability Statement

All data in manuscript.

TABLE 1. Set of primer sequence for evaluation of genetic expression of vitamin D receptor (VDR) gene in horses.

Gene	Accession No#	Strand	Primer sequence 5'3'
VDR	NM_001163959.1	F	ATCCTGACAGATGAGGAGGTG
		R	GAGACAAGGCAGGGATCTGA
β-actin	XM_014834961	F	GGAGTAAACGGATTGGCC
		R	CATGGGTGGAATCATACTGAAA

TABLE 2. Clinical criteria of horses with chronic inflammatory airway disease.

Clinical finding	Present	Not present
Poor performance	10/10	0/10
Abnormal respiratory rate	7/10 (70 %)	3/10 (30 %)
Nasal discharge	10/10 (100%)	0/10 (0 %)
Chronic cough	6/10 (100%)	4/10 (0 %)
Abnormal tracheal sound	10/10 (100%)	0/10 (0 %)
Abnormal lung sound	10/10(100%)	0/10 (0 %)

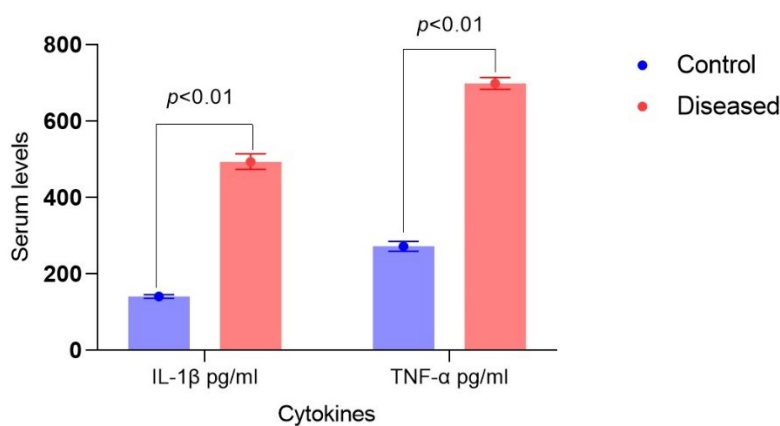


Fig. 1. Serum level of TNF-α and IL-1β in healthy horses and in those with chronic inflammatory airway disease.

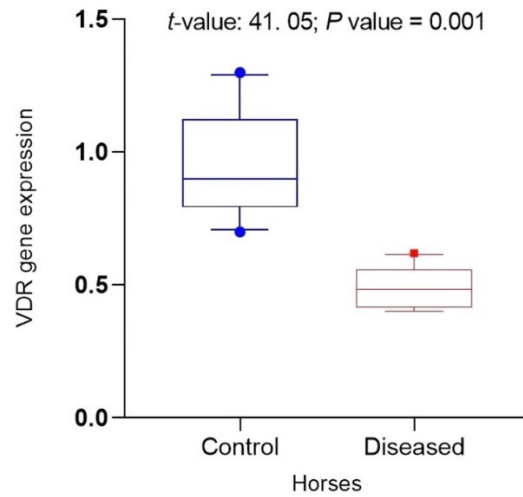


Fig. 2. Vitamin D receptor (VDR) gene expression pattern in healthy horses and in those with chronic inflammatory airway disease.

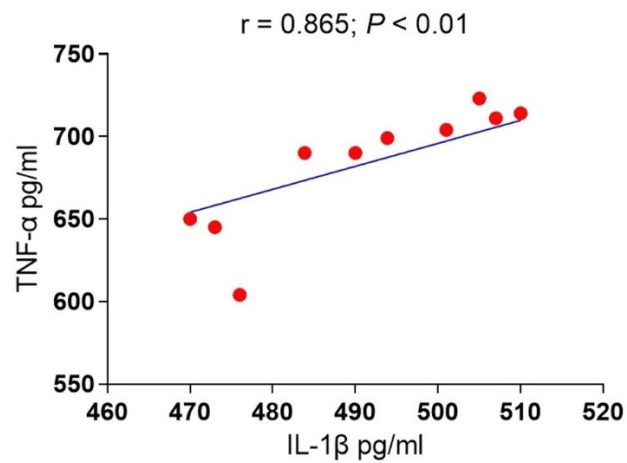


Fig. 3. Correlation between serum levels of TNF- α and IL-1 β in horses with chronic inflammatory airway disease.

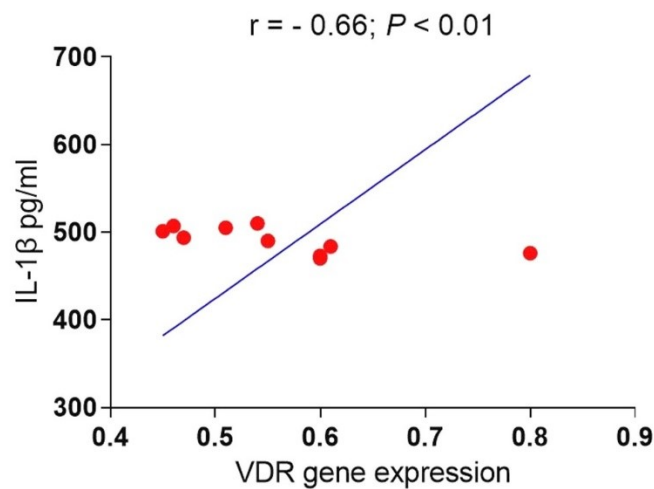


Fig. 4. Correlation between VDR gene expression and serum of IL-1 β level in horses with chronic inflammatory airway disease.

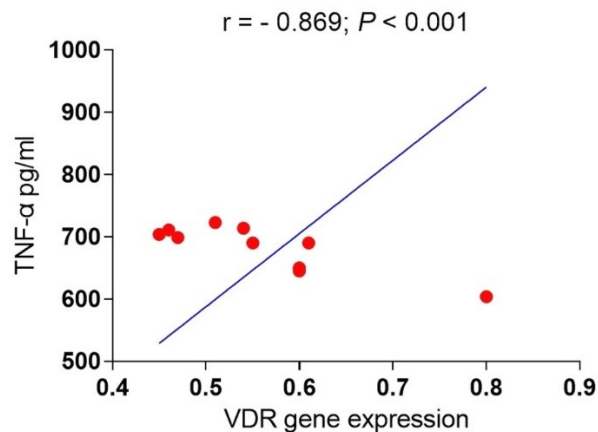


Fig. 5. Correlation between VDR gene expression and serum level of TNF- α in horses with chronic inflammatory airway disease.

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التعبير عن جين مستقبل فيتامين د (VDR) واستجابة السيتوكينات المرتبطة به في الخيول

العربية المصابة بمرض التهاب الشعب الهوائية المزمن

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يمثل مرض مجرى الهواء الالتهابي (IAD) حالات التهابية في الجهاز التنفسي السفلي. يحدث IAD في الخيول الرياضية وهو سبب شائع لانقطاع التدريب وضعف الأداء. كان الهدف من هذا البحث هو تقييم العلاقة بين جين مستقبل فيتامين د (VDR) والسيتوكينات في الخيول المصابة بـ IAD المزمن. تمت دراسة عشرة خيول مصابة بمرض AID وخمسة خيول تتمتع بصحة جيدة سريريًا. تم قياس مستويات مصل (TNF- α) والإنترلوكين-1 (IL-1 β)، وتم استخدام PCR في الوقت الحقيقي لتقدير نسبي لمستوى الاشارة لجين VDR في عينات دم الحصان. سريريًا، تعاني الخيول قيد الفحص من علامات سريرية مختلفة، بما في ذلك إفرازات مخاطية قيحية من الأنف، وزيادة معدل التنفس، والسعال المزمن، وأصوات الرئة والقصة الهوائية غير طبيعية. تم تسجيل زيادة كبيرة في مستويات TNF و IL-1 β في الخيول المصابة بمرض مجرى الهواء الالتهابي المزمن مقارنة بتلك الموجودة في الخيول السليمة سريريًا. ومع ذلك، انخفض التعبير الجيني VDR بشكل ملحوظ ($P > 0.001$) في الخيول المصابة بمرض مجرى الهواء الالتهابي المزمن مقارنة بتلك الموجودة في الضوابط. كان هناك ارتباط إيجابي بين مستويات TNF- α و IL-1 β ومع ذلك، لوحظ وجود علاقة سلبية بين التعبير الجيني VDR ومستويات IL-1 β و TNF- α . في الختام، تشير نتائج الدراسة الحالية إلى أن جين VDR له دور كبير في حدوث مرض IAD في الخيول. هناك حاجة إلى مزيد من الدراسات حول دور فيتامين د كعامل وقائي لـ IAD في الحالات الكلينيكية.

الكلمات الدالة: أمراض الجهاز التنفسي، الخيول، IL-1 β ، TNF- α ، فيتامين د.