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Вітамін D3 і α-токоферолу ацетат модифікують запальні та фіброзні процеси при системній склеродермії: преклінічні дані

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Системна склеродермія (ССД) — це хронічне автоімунне захворювання, що характеризується розвитком фіброзу і васкулопатій. Незважаючи на численні дослідження, наразі не існує ефективного лікування ССД. Ця робота мала на меті вивчити вплив α-токоферолу ацетату та вітаміну D3 на рівень сурфактантного протеїну D (SP-D), інтерлейкіну-13 (IL-13) і молекули адгезії судинних клітин-1 (VCAM-1) у доклінічній моделі ССД.

Дослідження включало інтактну групу (ІГ) (15 тварин) що не зазнали жодних втручань, контрольну групу (КГ) (20 тварин), яким вводили ізотонічний розчин, експериментальну групу №1 (ЕГ№1) (25 тварин), яким індукували ССД, вводячи їм підшкірно 0,5 мл 5% розчину гіпохлориту натрію (NaClO) тричі на тиждень протягом шести тижнів поспіль; та експериментальну групу №2 (ЕГ№2) (25 тварин), яким проводили корекцію шляхом ін'єкцій вітаміну D (1000 МО/100 г) та α-токоферолу ацетату (10 мг/100 г) внутрішньом'язово протягом 3 тижнів.

Сироваткові концентрації IL-13, SP-D і VCAM-1 були значно вищими в ЕГ№1 порівняно з контрольною групою (109.35 (93.23-199.05) та 8.50 (5.60-14.20), p=0.004; 490.20 (156.20-605.70) та 78.10 (40.80-100.40), p=0.004; 91.25 (85.00-264.98) та 19.50 (13.53-22.20), p=0.004 відповідно). Встановлено, що прийом вітаміну D3 і α-токоферолу ацетату позитивно впливає на всі три досліджувані параметри. Рівень SP-D в ЕГ № 2 був значно нижчим, ніж у ЕГ № 1 (490.20 (156.20-605.70) проти 123.75 (108.80-145.03), p=0.004). Концентрації IL-13 і VCAM-1 також були нижчими в ЕГ № 2.

У підсумку це дослідження надає докази сприятливого впливу вітаміну D3 і α-токоферолу ацетату на зниження рівнів SP-D, IL-13 і VCAM-1 у преклінічній моделі системної склеродермії.

Ключові слова: системна склеродермія, вітамін D, α-токоферолу ацетат, тваринні моделі.

Vitamin D3 and α-Tocopherol Acetate Ameliorate Inflammatory and Fibrotic Processes in Systemic Sclerosis: Preclinical Evidence

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Systemic sclerosis (SSc) is a chronic autoimmune disease characterized by fibrosis and vascular abnormalities. Despite extensive research, there is currently no effective treatment for SSc. This study aimed to investigate the effects of α-tocopherol acetate and vitamin D3 on the levels of surfactant protein D (SP-D), interleukin-13 (IL-13), and vascular cell adhesion molecule-1 (VCAM-1) in a preclinical model of SSc.

The study included an intact group (IG) (15 animals) with no interventions, control group (CG) (20 animals) injected with isotonic solution, an experimental group #1 (EG#1) (25 animals) that were induced with SSc by injecting them subcutaneously with 0.5 ml of 5% (NaClO) three times a week for six consecutive weeks; and experimental group #2 (EG#2) (25 animals) with correction provided by injections of vitamin D (1000 IU / 100 g) and α-tocopherol acetate (10 mg / 100 g) intramuscularly for 3 weeks.

The serum concentrations of IL-13, SP-D, and VCAM-1 were significantly higher in the EG#1 compared to the control group (109.35 (93,23-199.05) vs 8.50 (5.60-14.20), p=0.004; 490.20 (156.20-605.70) vs 78.10 (40.80-100.40), p=0.004; 91.25 (85.00 -264.98) vs 19.50 (13.53-22.20), p=0.004 respectively). The administration of vitamin D3 and α-tocopherol acetate was found to have a positive effect on all three parameters investigated. The SP-D level in the EG#2 was significantly lower than that in the EG#1 (490.20 (156.20-605.70) vs 123.75 (108.80-145.03), p=0.004). The concentration of IL-13 and VCAM-1 were also lower in the EG#2.

In conclusion, this study provides evidence of the beneficial effects of vitamin D3 and α-tocopherol acetate in reducing the levels of SP-D, IL-13, and VCAM-1 in a preclinical model of systemic sclerosis.

Keywords: Systemic sclerosis, vitamin D, α-tocopherol acetate, animal models.

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Introduction

Systemic sclerosis (SSc) is a complex autoimmune disorder marked by extensive fibrosis in tissues and internal organs, endothelial dysfunction leading to small vessel vasculopathy, and immunological aberrations. The etiology of this disease is currently unknown, although it is believed to result from a combination of genetic predisposition and environmental factors.

Respiratory system involvement is a common occurrence among patients with SSc, with interstitial lung disease (ILD) and pulmonary hypertension (PH) being the two most prevalent types of direct pulmonary involvement. In fact, over 70% of SSc patients suffer from pulmonary conditions, with interstitial lung disease and pulmonary hypertension being the most common [1]. Alarmingly, these two conditions account for 60% of SSc-related deaths [2]. Early pathological changes such as microvascular injury and alveolar inflammation have been implicated in the pathogenesis of SSc-related pulmonary diseases. Inflammation and autoimmune responses triggered by microvascular injury, either directly or indirectly, activate fibroblasts, leading to fibrosis [3]. Pulmonary microvascular endothelial cell injury is thought to result from hypoxia, inflammation, and endothelial-mesenchymal transition (EndoMT) [4]. Studies have shown that in patients with systemic sclerosis (SSc), there is an increased expression of vascular cell adhesion molecule-1 (VCAM-1) which is associated with vascular dysfunction and endothelial cell activation [5]. VCAM-1 is a cell surface molecule that is involved in the adhesion of leukocytes to endothelial cells and the subsequent migration of leukocytes into tissues.

Noteworty, specific biomarkers of lung fibrosis that could help predict the course of lung involvement are lacking. Since its discovery, surfactant protein D (SP-D) has emerged as a diagnostic and prognostic marker for ILD, particularly in the context of ILD-SSc [6]. A primary advantage of SP-D over classical markers of lung fibrosis in SSc is that it reflects pathological processes specific to the lungs. This is particularly important because cutaneous fibrosis peaks early and gradually improves, whereas lung fibrosis persists and worsens until the late stages of the disease [7]. SP-D is predominantly produced in the lungs, and its role in various forms of pulmonary fibrosis, including ILD-SSc, has been described in recent years [8]. Furthermore, increased SP-D levels have been also found to predict poor outcomes in patients with idiopathic pulmonary fibrosis [9].

In SSc, there is a disruption in the balance between extracellular matrix (ECM) accumulation and breakdown. The fibrosis associated with SSc results from the excess deposition of collagen, especially type 1 collagen [10]. Although the disease is characterized by both inflammation and fibrosis, the link between the two has come to light only recent decade. Inflammation and inflammatory mediators lead to excess ECM accumulation, resulting in scarring and fibrosis. Interleukin-13 (IL-13), a classic Th2 cytokine, is known to play a crucial role in fibrosis and is elevated in SSc [11]. SSc is considered a "Th2-polarized" disease, and several Th2 mediators may influence its course. Elevated level of IL-13 has been found in the serum of SSc patients compared to healthy controls. Additionally, genetic associations suggest that IL-13 is involved in SSc [11].

Even with considerable advances in understanding SSc, little progress has been made in treating the disease, and there is currently a lack of drugs capable of affecting its progression. Hence, this study aimed to investigate the effects of α-tocopherol acetate (vit E) and vitamin D3 (vit D) on the levels of SP-D, IL-13, and VCAM-1 in a preclinical model of SSc.

Materials and Methods

The following methodology is defined following the ARRIVE 2 updated guidelines for reporting animal research [12].

Experimental animals, their housing, care and monotoring

The adult Wistar rats weighing 220-240 g purchased from the Ivano-Frankivsk National Medical University (IFNMU) vivarium were used for this study. All experimental animals were housed under conventional conditions in the animal care facility by the IFNMU. The rats were kept in single-tier plastic cages with an upper hinged wall made in the form of a metal

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frame with a grid. The facility is well venti-lated and illuminated with an air temperature of +20-+24 C and a relative humidity of 45-65%. Daily housekeeping included changing the wood chip litter and washing the food dish. Once a week, the cages were disinfected with a 2% formalin solution.

All animal procedures were carried out in accordance with the bioethical principles of medical and biological experimental work declared in the provisions of the European Convention for the Protection of Vertebrate Animals used for Research and Other Scientific Purposes and approved by the IFNMU Ethics Commission, № 117/20 of 19.11.20.

Study design
We assembled our experimental animals into four groups: an intact group (IG) (15 animals) control group (CG) (20 animals), an experimental group #1 (EG#1) (25 animals) and experimental group #2 (EG#2) (25 animals). Group randomization was performed using the method of minimizing differences in weight. In order to exclude the influence of the daily rhythm and biological activity on the metabolism of rats, the sampling of the material was carried out in the morning, before feeding.

Blinding technic
To minimize potential errors in the interventions, the animals within each group were housed in the same cage. The intervention process could not be blinded as isotonic solution and sodium hypochlorite (NaClO) have distinguishable characteristics, while the control group received no interventions. However, each animal's blood sample and tissue specimen were labeled separately, so that the researchers were blinded to which group they belonged to. Additionally, to reduce statistical analysis bias, different groups were randomly assigned codes (such as group X, group Y, group Z, and group W).

Modeling of SSc
The induction of SSc was performed according to the following method (Fig.1): experimental animals from EG#1 were injected with 0.5 ml of 5% (NaClO) three times a week subcutaneously for 6 consecutive weeks as reported previously [13]. Isotonic solution was administered to laboratory rats of the CG by the same scheme and technic. The intact group did not receive any interventions. The laboratory animals from EG#2 in addition to NaClO, received a solution of vit E 10 mg / 100 g of body weight, intramuscularly and a solution of vit D 1000 IU / 100 g of body weight, intramuscularly for 3 weeks (second half of the experiment). All groups of laboratory animals were euthanized by decapitation 8 weeks after beginning of the experiment. The euthanasia was provided under thiopental anesthesia (thiopental was administered intramuscularly at a dose of 10 mg / 100 g rat weight).

Sample size
To determine the sample size for this pilot study, the "resource equation" method was
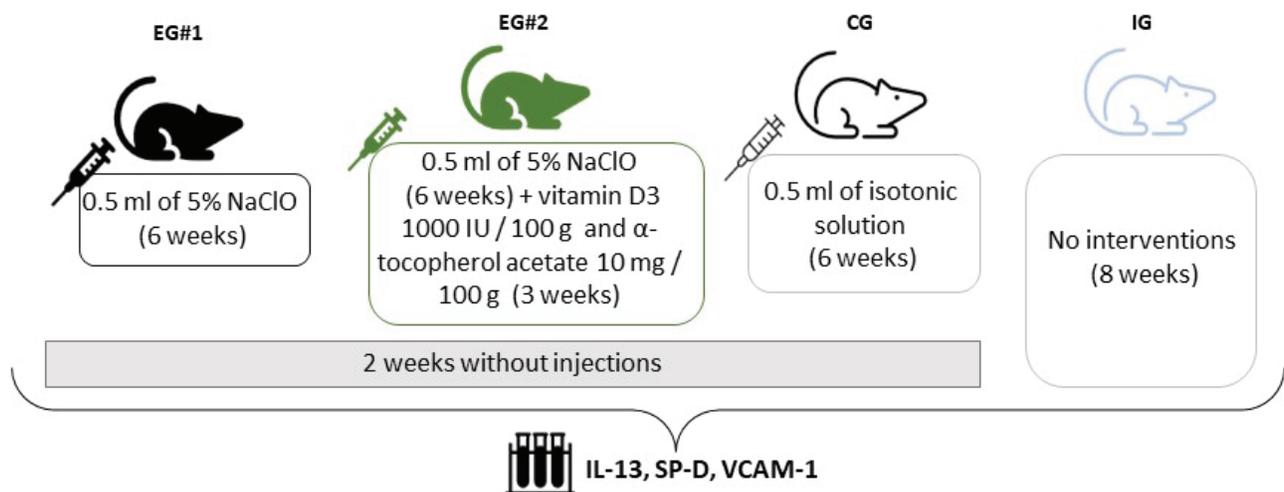

Figure 1. Graphic representation of the study design

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used. The equation used was   E = (15 x 3) - 3 = 42, which resulted in a sample size of 42. This sample size was deemed sufficient as it is greater than 20 [14]. Additionally, the expected attrition or death of animals was considered. Given the relatively long duration of the experiment, the corrected sample size for the experimental group was 25 animals, calculated as 15 divided by 0.6.

Reagents

Sodium hypochlorite (NaClO) solution was purchased from (PE "Latus", Kharkiv, Ukraine). The level of surfactant protein D (Elabscience SP-D, Texas, USA), the vascular cell adhesion molecule (Elabscience VCAM-1, Texas, USA), and interleukin 13 (Elabscience, Texas, USA) was determined by enzyme-linked immunosorbent assay. ELISA was performed on an enzyme-linked immunosorbent assay STAT FAX 303 plus.

Statistical analysis

The distribution of data was assessed using the Kolmogorov-Smirnov test. Descriptive statistics were reported as Me (IQR: Q1-Q3) (median with interquartile range) for non-normally distributed data. The Mann-Whitney test was used to analyze statistical differences between continuous variables. For comparisons among groups with three or more variables, the one-way nonparametric ANOVA (Kruskal-Wallis test) with pairwise comparisons was utilized. The statistical software used for data analysis was SPSS version 26.0 (SPSS Inc., Chicago, IL, USA).

Results

The levels of all three parameters did not differ between the control and intact groups (Table 1). However, in EG#1, the IL-13 (pg/ml) level was more than ten times higher than in the control group: 136.4 ± 109.35 (93.23-199.05) vs. 8.50 (5.60-14.20) (p=0.004). The serum concentration of SP-D (pg/ml) among the laboratory animals in EG#1 was significantly higher compared to the control group, and VCAM-1 serum concentration demonstrated the same tendency and was the highest in EG#1 (Fig. 2).

The administration of vitamin D and α-tocopherol acetate had a positive effect on all three investigated parameters. We found a statisti-
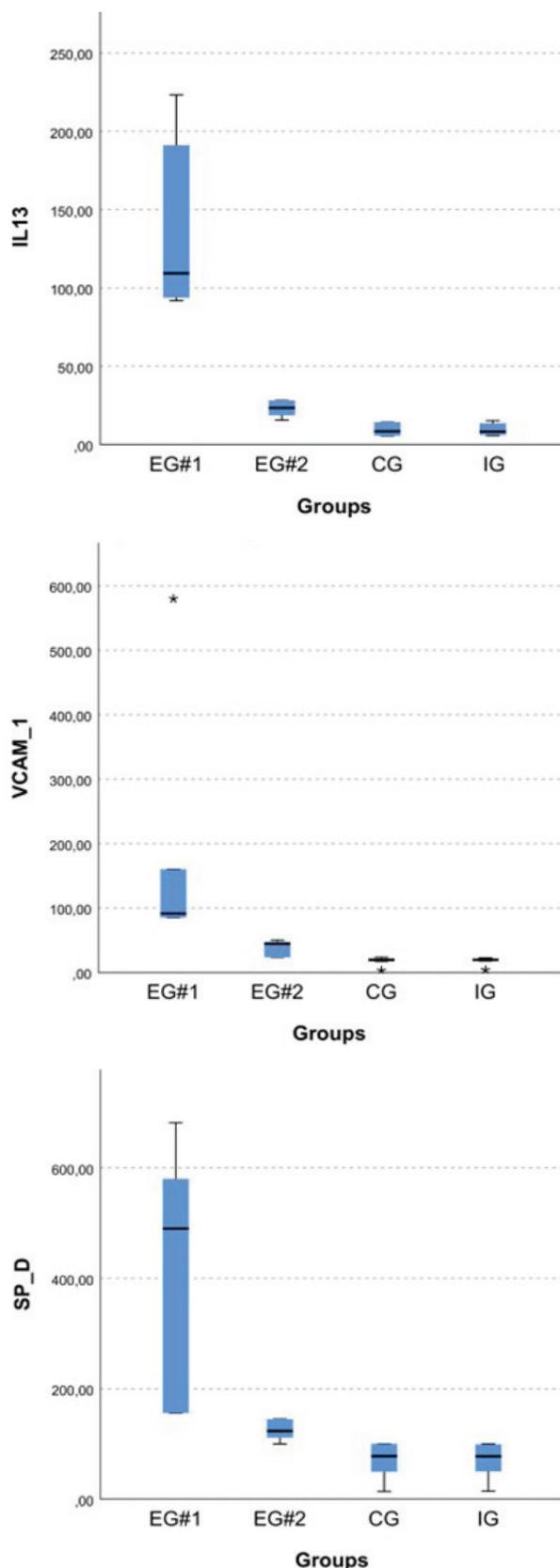

Figure 2. Laboratory values among four study groups

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Table 1

Effect of vitamin D3 and α-tocopherol on selected laboratory parameters

	Experimental group #1	Experimental group #2	Control Group	Intact Group
IL-13, pg/ml Me (IQR Q1-Q3)	109.35 (93,23-199.05)	23.40 (17.93-28.10)	8.50 (5.60-14.20)	8.25 (6.00-11.75)
VCAM-1, pg/ml Me (IQR Q1-Q3)	91.25 (85.00 -264.98)	44.05 (23.40-48,13)	19.50 (13.53-22.20)	18.20 (12.38-21.58)
SP-D, pg/ml Me (IQR Q1-Q3)	490.20 (156.20-605.70)	123.75 (108.80-145.03)	78.10 (40.80-100.40)	77.75 (41.43-90.90)

IL-13		VCAM-1		SP-D	
	P		P		P
EG#1-EG#2	0.004	EG#1-EG#2	0.004	EG#1-EG#2	0.004
EG#1-CG	0.004	EG#1-CG	0.004	EG#1-CG	0.004
EG#1-IG	0.004	EG#1-IG	0.004	EG#1-IG	0.004
EG#2-CG	0.004	EG#2-CG	0.006	EG#2-CG	0.006
EG#2-IG	0.004	EG#2-IG	0.004	EG#2-IG	0.004
CG-IG	0.748	CG-IG	0.749	CG-IG	0.872

cally significant difference between the two experimental groups regarding IL-13, SP-D, and VCAM-1 (Table 1). The most significant decrease after the administration of these vitamins was seen in IL-13 and SP-D levels, while the median level of VCAM-1 was decreased by two times.

Discussion
To the best of our knowledge, this is the first experimental study aimed at revealing the effects of combined therapy using vit D and E on laboratory animals with induced SSc-related pulmonary involvement. While vit D and α-tocopherol acetate have been separately studied as possible pathogenetic players in the management of SSc, data regarding their combined effect on the pulmonary system is limited. Our study showed that the combination of vit D and α-tocopherol acetate had a positive effect on the concentrations of IL-13, VCAM-1, and SP-D in the serum of laboratory animals with experimentally induced SSc.

Vitamin D has been found to possess immuno-modulatory, cardioprotective, and antifibrotic biological effects, which suggest it may be able to interfere with the pathophysiological mechanisms activated in SSc [15]. All three main branches of pathogenesis: autoimmunity, peripheral vasculopathy, and fibrosis might be aimed with vit D.

Vitamin D exerts pleiotropic effects by binding to and activating the vit D receptor (VDR), a nuclear receptor that manages vit D-responsive genes [16]. VDR is widely distributed throughout the body, which accounts for the multifaceted effects of vit D that extend beyond calcium and phosphate metabolism [17]. Vit D modulates innate and adaptive immunity by regulating the production of pro-inflammatory and anti-inflammatory cytokines [18] and inhibiting T cell proliferation [19]. Furthermore, vit D can interfere with the fibrotic process by regulating the TGFβ-pathway through SMAD-dependent transcription [20].

It is assumed that an immune system disorder is caused by an imbalance between pro-inflammatory T cells (Th17 or Th17.1) and regulatory T (Treg) cells [20]. Vitamin D can help restore this balance by stimulating Treg cells, thereby bringing the immune system back to a state of normalcy [21, 22]. This is why vit D is believed to have a protective effect against autoimmune diseases, due to its ability to modulate and promote immune tolerance.

Recent experimental studies have shown that VDR expression is decreased in SSc fibroblasts and in a murine model of skin fibrosis, which is dependent on TGF- β [23]. Additionally, VDR signaling can be activated by the vit

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D analog paricalcitol to inhibit TGF-β signaling and improve experimental fibrosis in the bleomycin model. Another study demonstrated that 17,20S(OH)2pD can: suppress total collagen synthesis, modulate key mediators in the TGF-β pathway, and alter expression of inflammatory cytokines in the bleomycin (BLM) model of skin fibrosis [24].

Clinical studies have provided evidence that vit D has the ability to inhibit both IL-17A and pro-fibrotic cytokines, indicating its potential anti-fibrotic effect [25]. These findings support the hypothesis that vit D supplementation in SSc patients may have a potentially beneficial effect on fibrotic processes by inhibiting the production of these cytokines. In turn, Caimmi et al. reported a correlation between low levels of vit D and an increased risk of developing digital ulcers in patients [26].

Vitamin E is a powerful intracellular antioxidant, which is known to prevent lipid peroxidation and protect polyunsaturated fatty acids in membrane phospholipids and plasma lipoproteins. It inhibits the synthesis of reactive oxygen species (ROS) and primarily functions as a peroxyl radical scavenger. Research by Ostojic and Damjanov revealed that patients with early diffuse cutaneous SSc who received vit E (400 IU/day) and ascorbic acid (1,000mg/day) for six months along with cyclophosphamide had a lower skin thickening progression rate than those on cyclophosphamide alone [27]. The upregulation of cytosolic phospholipase-A2 and cyclooxygenase-1 by vit E promotes the release of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation.

De Souza et al. conducted a study which demonstrated the effectiveness of a combination treatment involving pentoxyphylline and vit E for the treatment of fibrotic manifestations in systemic sclerosis [28]. The treatment was found to be effective within 16 weeks, and the reduction in MRSS persisted until the 24th week. The study also indicated that the combination treatment might influence the progression of ischemic ulcers in SSc. According to Fiori et al, topical application of vit E is beneficial in managing digital ulcers in SSc patients [29]. It is helping in reduction of healing time and provide faster relief from pain, while also resulting in significant cost savings.

SP-D is produced by type II alveolar cells in the lungs and plays a crucial role in maintaining proper pulmonary mechanical function. It is a commonly used serum marker for SSc-ILD [30]. Furthermore, its levels in the bloodstream may be indicative of the effectiveness of antifibrotic therapy [31]. Another study showed that the degree of decrease in SP-D levels during cyclophosphamide therapy could predict treatment response [32]. In turn, Ebata et al. suggested that a rapid decrease in SP-D levels in the good responder group could indicate a potent antifibrotic effect of RTX [33]. Therefore, the difference in SP-D levels between EG1 and EG2 could support the hypothesis that vit D and vit E have a positive influence on pulmonary remodeling.

IL-13 is a cytokine that is typically associated with Th2 cells and has been shown to stimulate the production of type I collagens, as well as the transition of fibroblasts into myofibroblasts [34]. Therefore, blocking Th2-associated cytokines could be a potential target for SSc therapy. Recent research suggests that IL-13 levels are linked to the severity of restrictive lung disease in SSc patients with early disease [11]. In addition, the IL-13/IL-33 axis is a relevant marker of disease activity in ILD for SSc patients with the diffuse form [35]. Moreover, in limited cutaneous systemic sclerosis (lcSSc) patients, IL-13 levels were observed to increase in those with pulmonary arterial hypertension (PAH) [34]. The expression of the IL-13 target gene MRC1 in circulating monocytes was also found to be correlated with pulmonary arterial pressure [36].

According to Greenblatt et al., the inflammatory subset of scleroderma is driven by IL-13 and may potentially benefit from IL-13 blockade, as evidenced by their comparison of human and murine scleroderma [37]. These findings suggest that therapies aimed at reducing these cytokines could effectively reduce collagen accumulation in SSc and prevent the development of chronic fibrosis. Our study has shown a decrease in IL-13 levels in the group of experimental animals that received vit D and vit E, indicating that these vitamins could serve as valuable additional therapies for immunological moderation.

As for VCAM-1, there are evidence that it plays important role in the microcirculatory damage in case of SSc [5]. Zanin-Silva et al. have shown

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that elevated levels of VCAM-1 are linked to vascular dysfunction and activation of endothelial cells in SSc patients [38]. Therefore, a decrease in the level of VCAM-1 can potentially indicate a decrease in the intensity of microcirculatory disorders, which could be a positive outcome for individuals with SSc.

In conclusions: This study provided evidence that administration of vitamins D3 and α-tocopherol acetate, given in combination, has a beneficial effect on IL-13, SP-D, and VCAM-1 levels in the organism of the experimental animals. These findings suggest that these vitamins could potentially be used as therapeutic agents for treating SSc. However, further studies are needed to confirm these findings in human subjects.

Study limitations
One of the limitations of this study is that it was conducted in an animal model, and therefore, the findings may not be fully generalizable to human patients with SSc. Second, the study only investigated the effects of vit D3 and α-tocopherol acetate on three parameters, namely SP-D, IL-13, and VCAM-1. Other factors involved in the pathogenesis of SSc were not investigated in this study. Therefore, it is unclear whether these vitamins can improve other disease-related parameters in SSc. Third, the study did not investigate the long-term effects of vitamin D3 and α-tocopherol acetate administration. Therefore, it is unclear whether these vitamins have any adverse effects or whether the beneficial effects observed in this study are sustained over time.

Conflict of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical statement
Approval was obtained from the local Ethics Committee (approval № 117/20 of 19.11.20). All experiments were performed following the bioethical principles of medical and biological experimental work asserted by the European Convention for the Protection of Vertebrate Animals used for Research and Other Scientific Purposes.

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