Vitamin D supplementation modulates autophagy in the pristane-induced lupus model

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Abstract

Introduction/objectives: Clinical evidence of skeletal muscle involvement is not uncommon in systemic lupus erythematosus (SLE). Because of the poor understanding of signaling pathways involved in SLE muscle wasting, the aim of this study was to evaluate the effects of vitamin D supplementation on skeletal muscle in mice with pristane-induced lupus.

Methods: Balb/c mice with lupus-like disease induced by pristane injection were randomized into three groups: pristane-induced lupus (PIL; n = 10), pristane-induced lupus + vitamin D supplementation (PIL +VD; n = 10) and healthy controls (CO; n = 8). Physical function was evaluated on days 0, 60, 120 and 180. The tibialis anterior and gastrocnemius muscles were collected to evaluate myofiber cross-sectional area (CSA) and protein expression.

Results: The PIL +VD group showed lower muscle strength compared to the CO and PIL groups at different time points. PIL mice showed similar myofiber CSA compared to CO and PIL +VD groups. LC3-II expression was higher in PIL compared to CO and PIL +VD groups. MyoD expression was higher in PIL mice compared to PIL +VD, while myostatin expression was higher in PIL +VD than PIL group. Myogenin expression levels were decreased in the PIL +VD group compared with the CO group. The Akt, p62 and MuRF expressions and mobility assessment showed no significance.

Conclusions: Changes in skeletal muscle in PIL model happen before CSA reduction, possibly due to autophagy degradation, and treatment with Vitamin D has an impact on physical function by decreasing muscle strength and time of fatigue. Vitamin D supplementation has a potential role modulating physical parameters and signaling pathways in muscle during pristane-induced lupus model.

Keywords: Systemic lupus erythematosus, Vitamin D, Skeletal muscle, Mice

Key points

- Clinical evidence of skeletal muscle wasting in SLE is not uncommon;
- To maintain the muscle homeostasis, a delicate balance between muscle protein synthesis and degradation is regulated by a network of interconnected pathways;
- The pristane-induced lupus model is likely to promote muscle atrophy due to increased autophagy,
which might be partially counterbalanced by vitamin D supplementation.

- The small impact on physical function at the end of the experiment is possibly related to the mild atrophy (about 10%) that the studied animal model developed.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease with clinical manifestations involving different organs and tissues [1]. SLE is characterized by autoantibody production, immune complex deposition, inflammation, and tissue damage. The incidence is 1–22 cases per 100,000 people per year, affecting mainly women of childbearing age [1].

Clinical evidence of skeletal muscle involvement, such as myositis and wasting, is not uncommon in SLE, and can lead patients to additional risk for muscle-related disability, risk of morbidity and mortality [5]. Patients with SLE experience lower dynamic muscle strength and decreased isokinetic muscle strength when compared to matched controls [2]. The lower extremity muscle strength is associated with physical disability scores and can predict significant declines in physical function, especially among the weakest patients [3]. Additionally, fatigue is another disabling aspect often related to SLE, that might be related to mitochondrial dysfunction in muscle tissue [4].

Excessive loss of muscle mass is associated with mortality and disability in several diseases [6]. In order to maintain muscle homeostasis, a delicate balance between muscle protein synthesis and degradation is regulated by a network of interconnected pathways (Fig. 1) [7]. Phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway stimulates muscle growth as an anabolic stimulus via the insulin-like growth factor 1 (IGF1) signaling [8]. In terms of muscle regeneration, satellite cells have an important role by leaving their quiescent state and turning on expression of myogenic lineage factors, such as myogenic factor 5 (Myf5), myoblast determination protein 1 (MyoD), myogenin, and myogenic regulatory factor 4 (Mrf4), to proliferate, differentiate and fuse with pre-existing fibers (Fig. 1) [9]. When the proteolytic systems are activated, contractile proteins and organelles are removed, resulting in the shrinkage of muscle fibers [6]. In addition, the myostatin pathway can trigger the Smad cascade, which inhibits Akt function, and is linked to muscle function by the stimulation of proteasomal and autophagic-lysosomal pathways [10].

The ubiquitin–proteasome system acts in the removal of sarcomeric proteins by upregulation of ubiquitin-protein ligases E3, such as muscle atrophy F-box (MAFbx) and muscle RING finger-1 (MuRF-1). The other main process of protein homeostasis is autophagy, which requires the expression markers for protein breakdown, such as microtubule-associated protein 1 light chain 3 (LC3), which contribute to autophagosome formation before the lysosomal degradation [10, 11]. Thus, muscle homeostasis by regulation of degradation/synthesis of protein content is a complex process and the impact of SLE on it has not been well studied. Vitamin D is considered a fat-soluble steroid hormone. In SLE patients, factors like reduced sun exposure due to photosensitivity, photo-protection use, renal vitamin D metabolism disruption and dark skin are explanations for vitamin D deficiency and insufficiency [13]. Studies reported that vitamin D deficiency leads to muscle weakness and a higher risk of falls, which vitamin D supplementation is able to reverse [26]. The vitamin D receptor (VDR) is widely expressed at classical sites of vitamin D activity, including intestines, parathyroid and bone, as well as at unclassical sites like immune cells and skeletal muscle cells. In skeletal muscle, VDR acts both as a nuclear and non-nuclear receptor, promoting increased Balb/c mice expression of myogenic factors and dose-dependently increase of intracellular muscle Ca²⁺ uptake in response to vitamin D supplementation, which may impact on muscle contraction [14–16]. Most SLE patients have vitamin D insufficiency (21–29 ng/mL) and deficiency (<20 ng/mL) [17]. Moreover, in SLE patients, vitamin D deficiency has been related to a higher degree of fatigue, and lower vitamin D levels were associated with disease activity scores [18–20].

Animal models may mimic SLE and are important tools for study its pathophysiology. Pristane-induced lupus (PIL) is a widely used model due to similarities with human SLE, including presence of arthritis, autoantibody production, glomerulonephritis, and elevated proinflammatory cytokines [21]. Because of our poor understanding of signaling pathways involved in SLE muscle wasting, the PIL model was used to investigate, and evaluate the possible impacts of vitamin D supplementation on muscle tissue.

Materials and methods

Mice

Eight weeks old female Balb/c mice were obtained from the Universidade Federal de Pelotas (Pelotas, RS, Brazil), and housed in the Animal Experimentation Unit of Hospital de Clínicas de Porto Alegre (Porto Alegre, RS, Brazil) in standard 12 h light/dark cycle, with controlled temperature (22 ± 2°C) and given water and food ad libitum. The present study was approved by Animal Ethics Committee (number 17–0011) and conducted following the National Institute of Health guidelines.
Experimental design and pristine treatment

Twenty-eight female Balb/c mice were randomly divided into three groups: control group (CO; n = 8); pristane-induced lupus group (PIL; n = 10); and pristane-induced lupus with vitamin D supplementation group (PIL + VD; n = 10). For the induction of lupus, 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich) was injected intraperitoneally (i.p) in PIL and PIL + VD mice. For the control group, 0.5 ml of 0.9% saline solution was injected i.p in CO mice. During the procedures, mice were anesthetized with 10% of isoflurane (Abbott Laboratório do Brasil Ltda., Brazil) and 90% of oxygen. Six months after the disease induction, mice were euthanized using an overdose of isoflurane; the tibialis anterior (TA) and gastrocnemius (GA) muscles were collected and stored for further analysis.
Disease development evaluation
The development of lupus-like disease in mice of the PIL group was assessed by presence of arthritis, cytokines profile alteration, serositis, hepatosplenomegaly, proteinuria, glomerular hypercellularity, and deposition of immune complexes, as previously published [22].

Vitamin D treatment
During the six months of the experimental period (day 0–180), mice were treated with vitamin D every two days. The hormonally active vitamin D metabolite, named 1,25-dihydroxy vitamin D3 (1,25(OH)2D3) or calcitriol (Calcijex, Abbott Laboratories), was diluted in PBS-Tween 20 (PBS-T) and subcutaneously injected in PIL+VD mice at a concentration of 2 µg/kg/day. CO and PIL mice were vehicle-treated with PBS-T.

Physical performance
Fatigue, muscle strength, mobility and physical performance were evaluated on days 0, 60, 120, and 180. The fatigue was measured on a treadmill, with an initial speed of 8.5 m/min for 9 min. Then the speed was increased by 2.5 m/min every 3 min, reaching a maximum of 45 m/min. Testing ended when mice met the criterion for exhaustion (remaining about 10 s in the rear of the treadmill), and the duration and distance reached were recorded [21].

The muscle strength test apparatus consisted of seven weights, made of stainless-steel wires, weighing 5, 20, 35, 50, 65, 80 g each. The mice were held by the middle/base of the tail and allowed to grasp the first weight for 3 s. When the 3 s holding was completed, the mice were submitted to the next heaviest weight. If the mice failed three times, the test was terminated, and the maximum time/weight achieved was assigned [23].

The mobility was evaluated in an acrylic movement box (Insight Equipamentos Ltda), where mice were placed individually. The spontaneous exploratory locomotion was detected by sensors located on the sides of the box and registered for 5 min. The movements were evaluated by a computerized trace system and the distance traveled and the average speed was determined.

Skeletal muscle histological evaluation
After anatomical dissection, the tibialis anterior muscle was fixed in 10% buffered formalin, paraffin-embedded and sectioned at 5 µm. The sections were stained with hematoxylin and eosin (H&E);10 images from each histological slide were captured with QI aging Digital Camera (Media Cybernetics). To determine the myofiber cross-sectional area (CSA), 20 myofibers of each image were measured with Image-Pro Express software (Media Cybernetics), totaling 200 measured myofibers per animal [24].

Protein expression
The GA muscle was used to quantify the protein expression of muscle markers of synthesis (Akt), regeneration (MyoD and, myogenin), and degradation (LC3B-II, p62, MuRF and myostatin) by Western blot. The muscle samples were homogenized with a lysis buffer and the homogenate’s protein concentration was determined by the Bradford assay. Muscle proteins were separated on 14–16% polyacrylamide gel electrophoresis, transferred to a PVDF membrane and, after blocking nonspecific sites, incubated overnight at 4 °C with the specific primary antibodies: Akt (1:1000), phosphorylated Akt (1:1000), MyoD (1:1000; Sigma, SAB4300397), myogenin (1:2000; Sigma, SAB2501587), LC3B-II (1:500; Cell Signaling, 2775), p62 (1:500; Cell Signaling, 5114) MuRF-1 (1:1000; Abcam, AB172479) and myostatin (1:1000; Abcam, AB203076). Then, primary antibodies were detected with a secondary antibody against rabbit, mouse, or goat by the chemiluminescence system. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used to normalize expressions. Raw data is available in the Additional file 1 attached. The densities of the specific bands were quantified with an imaging densitometer using Image J software.

Statistical analysis
Cross-sectional analyses. The Kolmogorov–Smirnov method was used to test for normality. Results are express as mean ± standard deviation (SD), median (interquartile range), and number (%) as appropriate. Independent-samples Kruskal–Wallis test, one-way ANOVA test followed by Tukey test.

Longitudinal analyses. Generalized estimating equations (GEE) were performed using a linear or Poisson log-linear model, as appropriate, for comparison between groups during the experimental period. Results are expressed as estimated mean and standard error (SE). The significance level was set at $p \leq 0.05$ for all analyses. Statistical analyses were performed in PASW 18.0 Statistics for Windows.

Results
The PIL group developed the disease as expected and presented a proinflammatory cytokine profile
As previously described by our group, the PIL mice developed arthritis with progressively increased severity over time. Additionally, at the end of the experimental period, PIL mice showed alteration in cytokine profile, with higher IL-6, TNFα and IFN-γ levels compared to
CO mice, serositis, hepatosplenomegaly, proteinuria, glomerular hypercellularity, and immune complexes deposition [22].

**Vitamin D treatment modulates muscle strength during the experimental period**

Although a transitory decreased muscle strength was observed for the PIL + D on day 60, at the end of the experiment both PIL and PIL + D, on day 120 the difference between groups was not maintained, and the groups had similar muscle strength measures ($p > 0.05$; Fig. 2a).

**Vitamin D treatment affects fatigue time at different time points**

PIL + VD mice took less time to fatigue when compared with the CO group at day 60 ($p = 0.001$; Fig. 2b), while there was no significant difference in fatigue time between PIL and PIL + VD groups (Fig. 2b). On days 120 and 180, the PIL + VD group showed significantly lower fatigue time than the CO group ($p = 0.004$; $p = 0.03$, respectively). The PIL group also presented a decreased time of fatigue when compared with the CO group at day 180 ($p = 0.001$; Fig. 2b), but there was no significant difference in fatigue times between PIL and PIL + VD groups ($p = 0.893$; Fig. 2b). Additionally, the PIL group presented a similar on days 60 and 120 compared with the CO group ($p = 0.070$, $p = 0.063$, respectively). The exploratory locomotion test did not show differences in mobility rates among the groups (Fig. 2c).

**Treatment with vitamin D modulates protein degradation markers on muscle without differences in muscle mass**

When the skeletal muscle was analyzed, the CSA means did not differ between the groups (Fig. 3a and b). The next step was to analyze the degradation markers of protein breakdown. The LC3B-II expression (autophagy marker) was significantly higher in the PIL group compared to CO ($p = 0.049$) and PIL + VD groups ($p = 0.014$; Fig. 4a). Myostatin expression increased in the PIL + VD group compared to the PIL group ($p = 0.012$; Fig. 4b). Regarding the expression of p62 and MuRF-1, both showed no statistical difference among the analyzed groups (Fig. 4c and d, respectively). Markers of protein synthesis (Akt) and regeneration (MyoD and myogenin) were analyzed.

![Graph A](image1.png)

**Fig. 2** Muscle strength, fatigue, and mobility during the experimental period. Measures of (a) muscle strength, (b) fatigue and (c) mobility in mice from CO, PIL and PIL + VD groups. Data represents MEAN ± SEM values. a muscle strength: ***$p < 0.0001$ CO vs PIL + VD; **$p < 0.01$ PIL + VD vs PIL; *CO vs PIL + VD, b fatigue: ***$p < 0.0001$ CO vs PIL + VD and PIL + VD vs PIL; *$p < 0.05$ PIL + VD vs CO
in this study; the PIL group presented higher levels of MyoD expression than the PIL+VD group \((p = 0.029, \text{Fig. 5a})\), and myogenin expression was decreased in the PIL+VD group when compared with the CO group \((p = 0.013; \text{Fig. 5b})\). For Akt expression, there was no difference in phosphorylated Akt and Akt total among the groups \((\text{Fig. 6a and b})\); in addition, the ratio Akt/phosphorylated Akt \((\text{pAkt})\) showed no statistical difference among the groups studied \((\text{Fig. 6c})\).

**Discussion**

The main findings of this study were: (1) vitamin D supplementation did not have a significant impact on skeletal muscle of this experimental model. In fact, vitamin D increased fatigue; (2) in this lupus experimental model, the impact on muscle area, fatigue and strength was limited \((3)\) vitamin D had some beneficial effect on autophagy, but it does not appear to influence muscle function significantly.

The muscle was analyzed molecularly by signaling pathways related to protein synthesis, regeneration, and degradation processes and performance by physical parameters. Mice mobility was not different among our experimental groups over time, while vitamin D did not beneficially modulate the muscle strength and fatigue of the mice. It has been reported that muscle weakness is often present in SLE patients, and vitamin D supplementation has been shown to have a positive impact on total body strength, specifically on the lower limbs \([2, 4, 5, 25]\). Perhaps the duration and intensity of inflammatory response in the studied model was not enough to induce the changes observed in clinical practice, or the mechanisms involved in muscle weakness in SLE patients are not reproduced in this model.

On days 60 and 180 we observed higher fatigue in PIL and the PIL+VD compared to the CO animals. Several studies have reported that SLE patients present higher fatigue levels than matched controls, and vitamin D supplementation during 3, 6, and 12 months reduced fatigue, although one study described no association between fatigue and circulating levels of 1,25(OH)2D37 \([2, 3, 26, 27]\).

The changes in skeletal muscle area observed in this study were mild compared to chronic arthritis models, that presented a 30–41% reduction in muscle CSA in previous studies, or to osteoarthritis model, which showed a 10% reduction in GA area compared to controls \([23, 28, 29]\). In patients with established SLE, type II fibers atrophy has been found, compared to controls, although the atrophy degree was not described \([30]\). Also, type II atrophy in SLE may be a potential cause of muscle symptoms irrespective of the time of biopsy, treatment received, and presence or absence of myositis \([30]\). Our experimental lupus model and the supplementation with vitamin D had no impact on muscle. On the other hand, vitamin D administration has been linked to muscle improvement.
Fig. 4 Protein expression of degradation markers in gastrocnemius muscle. Expression of (a) LC3, (b) myostatin, (c) p62 and (d) MuRF-1 in CO, PIL and PIL+VD groups. Data in (a) and (b) represent mean ± SD *p<0.05.

Fig. 5 Protein expression of regeneration markers in gastrocnemius muscle. Expression of (a) MyoD and (b) myogenin in CO, PIL and PIL+VD groups. Data in (a) and (b) represent mean ± SD *p<0.05.
by increasing CSA in the tail suspension model [25, 31], were the main mechanism of muscle atrophy is immobility. Also, hemodialysis patients receiving calcitriol or paricalcitol presented larger thigh muscle CSA, evaluated by magnetic resonance imaging, than control individuals [31]. As the muscle area of the diseased mice did not change during the 6 months of the experimental period, we hypothesized that the atrophy may take longer to occur, and the increased fatigue could be the earliest finding. Longer observations periods might be necessary for significant muscle atrophy to occur.

The normalization in autophagy rates after treatment with vitamin D was the only positive aspect of the treatment. Autophagy, a lysosome-mediated catabolic process, is responsible for the degradation of numerous damaged cytoplasmic constituents in response to stress, starvation, and growth factor deprivation [32–34]. As a major cellular process, it involves several protein interactions to polyubiquitylated substrates to deliver them to autophagosomes as a degradation signal, like p62 binding to LC3B-II on the growing autophagosome membrane to activate protein breakdown [35]. Thus, LC3B-II expression reflects the number of autophagosomes, since it localized from phagophore to lysosomal degradation [36]. LC3B-II expression was increased in the PIL mice muscle compared to both the CO and PIL + VD groups. On the other hand, p62 expression did not differ between groups, possibly because p62 is not strictly needed for autophagosome formation [37]. The absence of p62 expression in all groups, and the increased expression of LC3B-II in the PIL group, are possibly related to proteolysis in a non-ubiquitylated fashion, which can occur in an early phase of protein breakdown [38].

Moreover, vitamin D supplementation probably played a role by inhibiting the autophagy process caused by pristane-induced disease, since PIL + VD mice had muscle LC3B-II expression comparable to CO mice. In models of acute myocarditis and myocardial injury, vitamin D supplementation has already been described as a regulator of autophagosome formation [39, 40].

Fig. 6  Protein expression of synthesis markers in gastrocnemius muscle. Expression of (a) Akt, (b) phosphorylated Akt and (c) phosphorylated Akt/akt total ratio in CO, PIL and PIL + VD groups. Data in (a) and (b) represent mean ± SD while data in (c) represent median (interquartile range). *p < 0.05
In the pathogenesis of SLE, changes in autophagy maybe related to the increased IFN-α levels [41]. IFN-α increase can induce the autophagy death via JAK/STAT3, PI3K/AKT/mTOR, and MAPK pathways in multiple cancer cell lines, potentiating its functions in antigen presentation, proliferation inhibition, as well as being a positive feedback loop for its production [42]. Our group has demonstrated that PIL mice present higher levels of IFN-1 in serum samples, which could contribute to the augmented autophagy process in muscle tissue and increased protein breakdown [21]. To verify if autophagy is activated by PI3K/AKT/mTOR signaling, we assessed Akt content in GA muscle. Akt expression showed no difference among our experimental groups, even following vitamin D administration. Akt controls protein degradation by phosphorylating and sequestering FOXO family transcription factors in the cytoplasm, thus inhibiting their translocation to the nuclei to interact with target genes, such as MAFbx and MuRF-1 [43]. In vitro data show that human myoblasts treated with 1,25(OH)2D3 activate Akt and inhibit MAFbx and MuRF-1 expression [44]. Also, in a vitamin D-deficiency model, higher muscle expression of the ubiquitin-conjugating enzyme has been shown, while vitamin D administration reversed this change [45]. Perhaps the increase in Akt and MuRF-1 content in muscle tissue occurs before the autophagy process initiates since Akt can be upregulated and phosphorylated more rapidly to trigger muscle wasting.

We also investigated if satellite cell activation is impaired in the PIL model. The PIL mice showed higher expression of MyoD than PIL + VD mice, indicating signs of simultaneous muscle damage and repair; thus, myogenin expression was lower in the PIL + VD group than in the CO group. Another indicator of disease-mediated anabolic compensation was the reduced myostatin expression in the PIL group compared to the PIL + VD group. Similar data was reported in the arthritis model, in which both satellite cell activation and myostatin levels were decreased [46]. In fact, in our previous study, the PIL mice present increased circulating levels of IFN-γ, IL-6, and TNF-alpha compared with the PIL + VD mice, which could be related to higher MyoD, and decreased myostatin expression [22]. Increased MyoD content allows the satellite cell proliferation and the up-regulation of myogenin, thereby enabling myoblast differentiation, whereas myostatin negatively regulates muscle growth via decreased myogenesis [47]. Indeed, we found decreased LC3-II, Myod, and myogenin alongside an increase of myostatin in muscle tissue of treated animals. As a systemic treatment, vitamin D does not interact directly with muscle tissue, but it seems to decrease LCB-II which is a protein critical to the autophagosomal formation, so we consider a positive impact during the treatment. However, autoimmune diseases such as SLE have a high level of pro-inflammatory cytokines, which can modulate muscle tissue and dysregulated protein synthesis [46]. Myostatin levels were decreased in the PIL group, which can be a compensatory response during the development of the disease similar to what happens in inflammatory arthritis [46] and the treatment with vitamin D modulates the increase of myostatin as a way to counterbalance the lack of autophagy degradation characteristic by low LC3-II levels. The same can happen with proteins responsible for myoblast differentiation and proliferation, such as MyoD and Myogenin [48]. Also, this modulation can be a response to a lack of activation of the degradation process needed to mature healthy muscle cells.

The present study has limitations, such as the lack of proinflammatory and anti-inflammatory cytokines evaluation in the skeletal muscle tissue, which would enable the assessment of vitamin D impact on local cytokine profile. The vitamin D receptor evaluation would permit a better description of the vitamin D activity following the PIL model and Vitamin D levels measured and evaluated across the experiment to understand its role during Vitamin D insufficiency and muscle wasting.

**Conclusion**
The PIL model presented increased fatigue and autophagy, and treatment with vitamin D appear s to correct the increased autophagy, although no beneficial function effect could be observed. Absence of significant muscle atrophy in the studied model suggest that muscle involvement is light or take longer to occur.

**Supplementary Information**
The online version contains supplementary material available at https://doi.org/10.1186/s42358-022-00261-4.

**Additional file 1**. Raw data related to protein expression of AKT, Myostatin, p62, MuRF-1, MyoD and myogenin with the respective housekeeping protein GAPDH.

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Author contributions
All authors participated in the design of the study. Statistical analyses were conducted by M.S, R.C.E.S and J.M.S.S. All authors contributed to interpretation of the results. B.J.B, J.M.S.S, R.C.E.S, E.F. contributed to the drafting of the manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content, approved the final version and are accountable for the integrity of its content.

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Availability of data and materials
The data that support the findings of this study are available at Universidade Federal do Rio Grande do Sul and Additional file 1 can be found in the Additional file 1.

Declarations

Ethical approval and consent to participate
The present study was approved by Animal Ethics Committee (number 17-0011) and was conducted in accordance with the National Institute of Health guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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