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VITAMIN D SUPPLEMENTATION ATTENUATES ACUTE INFLAMMATORY RESPONSE

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ABSTRACT: This study evaluated the effects of daily vitamin D supplementation on the acute inflammatory response in experimental model by different phlogistic agents: carrageenan, prostaglandin and dextran. Animals (rats) orally received (gavage) a single dose of vitamin D or daily supplementation for 7, 15 or 30 days prior to paw edema induced. Vitamin D supplementation for 15 and 30 days significantly reduced the carrageenan-induced inflammatory process, which could be at least partially explained by the reduction of tumor necrosis factor α levels (TNFα). Results indicate that vitamin D supplementation may be a useful therapeutic adjuvant for controlling the acute inflammatory process.

KEY WORDS: Anti-inflammatories; Cholecalciferol; Inflammation; Vitamin D.

SUPLEMENTAÇÃO COM VITAMINA D ATENUA A RESPOSTA INFLAMATÓRIA AGUDA

RESUMO: Este estudo avaliou os efeitos da suplementação diária de vitamina D na resposta inflamatória aguda em modelo experimental por diferentes agentes flogísticos: carragenina, prostaglandina e dextrana. Os animais (ratos) receberam por via oral (gavagem), dose única de vitamina D ou suplementação diária durante 7, 15 ou 30 dias antes da indução do edema de pata. A suplementação com vitamina D por 15 e 30 dias reduziu significativamente o processo inflamatório induzido por carragenina, o que poderia ser explicado, pelo menos parcialmente, pela redução dos níveis de fator de necrose tumoral α (TNFα). Os resultados indicam que a suplementação de vitamina D pode ser um útil adjuvante terapêutico para o controle do processo inflamatório agudo.

PALAVRAS-CHAVE: Anti-inflamatórios; Colecalciferol; Inflamação; Vitamina d.

INTRODUCTION

Vitamin D is known to be involved in regulating calcium homeostasis, bone formation, and resorption. However, recent studies have reported the benefits of vitamin D in different clinical conditions, such as diabetes, asthma, rheumatoid arthritis, atopic dermatitis, and cardiovascular disease, among others. These studies found that the actions of vitamin D go beyond calcium metabolism and bone formation, with important involvement in the immune system[1,2,3].

Vitamin D receptors have been identified in a wide array of previously unrelated tissues, such as the brain, heart, skin, intestine, gonads, prostate, breasts, bones, kidneys, and parathyroid glands[1,2,3]. The microsomal enzyme 1α-hydroxylase (CYP27B1) belongs to the cytochrome P450 superfamily. It is capable of converting vitamin D into an active form in various tissues, thus seemingly contributing to its effects[4].

Some *in vitro* studies found that vitamin D has immunomodulatory activity and participates in the production of cytokines and components that are related to the inflammatory process, such as growth factors, nitric oxide (NO), and metalloproteinases[3,5,6,7].

De Castro (2011)[3] found a positive correlation between a decrease in serum concentrations of calcidiol (i.e., an inactive metabolite of vitamin D) and increases in the production of T cells that were autoreactive against the organism and synthesis of proinflammatory cytokines (IL-12 and interferon-γ). According to some authors, vitamin D_3 supplementation appears to reduce the rate of development of many inflammatory diseases[8,9].

The mechanism by which vitamin D plays a role in the inflammatory process is not well understood.[6] Most studies reported beneficial effects of this vitamin on the chronic inflammatory process[2,5,9,10]. No studies of which we are aware have reported its effects on the acute inflammatory response.

In the present study, we investigated the effect of vitamin D supplementation on the experimental acute inflammatory response that was induced by different phlogistic agents.

METHODOLOGY

ANIMALS

Male Wistar rats, weighing 200-220 g, were kept under controlled temperature (22°C ± 2°C) and a 12 h/12 h light/dark cycle with food (feed Nuvilab®) and water available *ad libitum*. The experimental protocols were approved by the Committee on the Use of Animals of the State University of Maringá (CEUA/UEM 9504220615).

PAW EDEMA INDUCTION

The animals received an intradermal injection in the left hind paw of 100 μl of the following phlogistic agents, dissolved in 0.9% saline: carrageenan (Cg; 200 μg/paw), prostaglandin E_2 (PGE_2; 0.1 μg/ml), and dextran (Dx; 300 μg/paw). The same volume of 0.9% saline was administered in the right hind paw. The volume of both paws was determined using digital plethysmography 1st, 2nd, and 4th hours after the injection of Cg and PGE_2, and 30, 60, 120, and 240 min after the injection of Dx.[14] The increase in final paw volume (μL x 10) was calculated by subtracting the volume of the paw that received saline (control paw) from the volume of the paw that received the phlogistic agent.

ANIMAL TREATMENT

Vitamin D_3 (cholecalciferol; Zhejiang Garden Biochemical High-tech Co.; lot no. C201412011A-3) was diluted in a suspension of 91% carboxymethylcellulose (CMC) + 9% olive oil. This combination that comprised the vehicle was chosen because it is innocuous and facilitates the dissolution of vitamin D. The animals were orally treated (gavage) with vitamin D_3 at doses of 0.5 mg/kg body weight (~4,000 IU) or 1 mg/kg body weight (~8,000 IU).[11] The animals were randomly assigned to the following groups:

- **group I** (Cg only; control) – (n=7)
- **group II** (PGE_2 only; control) – (n=7)
- **group III** (Dx only; control) – (n=7)
- **group IV** (single oral dose of 1 mg/kg vitamin D 1 h

before the induction of paw edema by Cg [Cg + Vit $D_{1.0}$ - SD]) – (n=7)

- **group V** (supplemented daily with 1 mg/kg vitamin D for 7 days prior to the induction of paw edema by Cg [Cg + Vit $D_{1.0}$ – 7d]) – (n=7)
- **group VI** (supplemented daily with 1 mg/kg vitamin D for 15 days prior to the induction of paw edema by Cg [Cg + Vit $D_{1.0}$ – 15d] – (n=7)
- **group VII** (supplemented daily with 1 mg/kg vitamin D for 30 days prior to the induction of paw edema by Cg [Cg + Vit $D_{1.0}$ – 30d]) – (n=7)
- **group VIII** (supplemented daily with 0.5 mg/kg vitamin D for 15 days prior to the induction of edema by Cg [Cg + Vit $D_{0.5}$ – 15d]) – (n=7)
- **group IX** (supplemented daily with 0.5 mg/kg vitamin D for 30 days prior to the induction of edema by Cg [Cg + Vit $D_{0.5}$ – 30d]) – (n=7)
- **group X** (supplemented daily with 1.0 mg/kg vitamin D for 15 days prior to the induction of edema by PGE_2 [PGE_2 + $VitD_{1.0}$ - 15d]) – (n=7)
- **group XI** (supplemented daily with 1.0 mg/kg vitamin D for 15 days prior to the induction of edema by Dx [Dx + $VitD_{1.0}$ - 15d]) – (n=7)

Additional groups of animals were orally treated with the reference drugs naproxen Sigma-Aldrich® (NPX; 3 mg/kg– (n=5) and cyproheptadine Sigma-Aldrich® (Ci-Hep; 10 mg/kg– (n=5) 1 h before the induction of edema and with the vehicle (CMC + olive oil) for 30 days.

PREPARATION OF PLANTAR TISSUE

Four hours after carrageenan-induced paw edema, the animals were sacrificed with Isoflurane 5% + CO_2 by inhalation route. The plantar tissue of the paw that was injected with the phlogistic agent was removed and placed in a centrifuge microtube that contained 0.5 ml of 4 mM phosphate-buffered saline (PBS), pH 5.4. The sample was then homogenized and centrifuged at 6000 × *g* at 4°C for 20 min. The supernatant was used for the analyses below.

DETERMINATION OF MYELOPEROXIDASE ACTIVITY

Myeloperoxidase (MPO) activity was determined in the supernatant of the plantar tissue homogenate according to Rocha et al. (2014).[12] An aliquot (10 μL) of the supernatant was placed in triplicate in a 96-well microplate, to which was added 50 mM PBS solution (pH 6.0) that contained 0.19 mg/ml *O*-dianisidinedihydrochloride and 0.0005% H_2O_2. The reaction was quenched with 1.46 M sodium acetate solution (pH 3.0), and MPO activity was determined by the end-point technique by reading absorbance at a wavelength of 460 nm.

DETERMINATION OF NITRITE CONCENTRATION

The concentration of nitrite was determined by the Griess method[13], which indirectly determines the concentration of NO in tissue samples. Supernatants (50 μl) of plantar tissue were placed in triplicate in a 96-well microplate, to which Griess solution was added at room temperature. After 10 min, readings were performed using an enzyme-linked immunosorbent assay (ELISA) reader at 550 nm. Nitric oxide concentrations were calculated from a standard curve of sodium nitrite. The results are expressed as μM/ml.

DETERMINATION OF TUMOR NECROSIS FACTOR α CONCENTRATION

Tumor necrosis factor α (TNF-α) concentrations were determined in homogenate supernatants (100 μL) of plantar tissue by spectrophotometry using the ELISA technique according to the manufacturer's instructions (Rat TNF-α Platinum Elisa 96 test, Ebioscience®).

ASSESSMENT OF BODY WEIGHT GAIN

Body weight gain was evaluated on alternate days throughout the vitamin D supplementation period. In this experiment, the animals were randomly assigned to a normal group (without treatment), vehicle group (treated with vehicle [CMC + olive oil]), and groups that received vitamin D supplementation (1 mg/kg) for 30 days.

STATISTICAL ANALYSIS

Statistic 8.0 software (StatSoft®, Palo Alto, CA, USA) was used for the statistical analysis. The paw edema data were examined for assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). Since data followed normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test), we analyzed using repeated-measures analysis of variance (for paw edema), or one-way ANOVA (for MPO, NO and TNFα tests). If a main effect of group was found, Tukey-Kramer's multiple range test was used to distinguish between groups. The results are expressed as mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant.

RESULTS

VITAMIN D SUPPLEMENTATION REDUCED CARRAGEENAN-INDUCED PAW EDEMA

The intradermal injection of carrageenan significantly increased the volume of the injected paw in the Cg group at the 1st, 2nd, and 4th hours after application of the phlogistic agent (22.7 ± 1.7 ($p<0.01$), 40.9 ± 3.3 ($p<0.01$), 59.3 ± 3.5 ($p<0.01$), respectively). Treatment with vehicle (CMC + olive oil) for 30 days did not affect the development of the inflammatory response (26.3 ± 3.2, 42.8 ± 3.2, 62.4 ± 2.7, respectively).

Treatment with a single dose of vitamin D (1 mg/kg) 1 h before induction of the inflammatory response did not affect the development of paw edema at any of the periods of evaluation (Cg + $VitD_{1.0}$ - SD: 23.9 ± 2.3 ($p=1.00$), 44.4 ± 2.3 ($p=1.00$), and 55.9 ± 4.5 ($p=0.99$), respectively) compared with the Cg group. Similarly, daily supplementation with vitamin D (1 mg/kg) for 7 days did not significantly affect the evolution of paw edema in the same periods of evaluation (Cg + $VitD_{1.0}$ - 7d: 18.5 ± 1.2 ($p=0.99$), 38.8 ± 2.5 ($p=1.00$), and 55.8 ± 2.3 ($p=0.99$), respectively).

Daily supplementation with vitamin D (1 mg/kg) for 15 days significantly reduced edema at the 1st, 2nd, and 4th hours (Cg + $VitD_{1.0}$ - 15d: 13.6 ± 1.1 ($p<0.05$), 27.9 ± 1.7 ($p<0.05$), and 41.2 ± 1.7 ($p<0.01$), respectively). Daily supplementation with vitamin D (1 mg/kg) for 30 days significantly reduced the development of edema (Cg + $VitD_{1.0}$ - 30d: 12.9 ± 1.8 ($p<0.05$), 32.2 ± 1.5 ($p<0.05$), and 45.8 ± 1.9 ($p<0.01$), respectively). Treatment with NPX significantly reduced paw edema only at the 4th hour ($p<0.01$) (Figure 1A).

Considering the inhibitory effect of vitamin D (1 mg/kg) on carrageenan-induced paw edema following vitamin D supplementation for 15 and 30 days, further experiments were conducted to evaluate the effects of a lower dose of vitamin D (0.5 mg/kg). This treatment protocol did not result in a significant reduction of the evolution of edema at any of the periods of evaluation (Figure 1B).

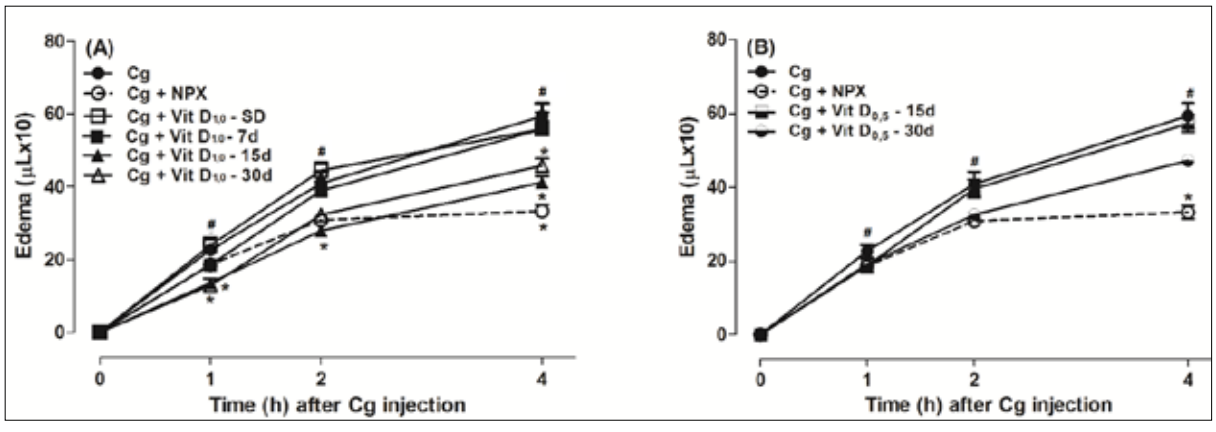

Figure 1. Effect of vitamin D (**A**) given orally in a single dose of 1 mg/kg (Cg + Vit $D_{1.0}$ - SD) or supplemented daily for 7, 15 and 30 days at 1 mg/kg (Cg + Vit $D_{1.0}$ – 7d, 15d, and 30d) and (**B**) supplemented daily for 15 and 30 days at 0.5 mg/kg (Cg + Vit $D_{0.5}$ – 15d and 30d) on the development of paw edema induced by a carrageenan (Cg) injection in Wistar rats. Cg untreated control animals that received carrageenan. The positive control group was treated with oral naproxen at a dose of 3 mg/kg (Cg + NPX). The data are expressed as the mean paw volume ± SEM 1, 2, and 4 h after the Cg injection. $^{\#}P < 0.05$ compared 1st, 2nd, and 4th hours after paw edema induced; $^{*}p < 0.05$, compared with Cg group (repeated-measures analysis of variance followed by Tukey's test - Statistic 8.0 software - StatSoft®).

No significant differences of inhibitory effect on paw edema were found between groups that received daily supplementation with vitamin D (1 mg/kg) for 15 and 30 days. Thus, we adopted the 15-day supplementation period to evaluate the effects of vitamin D in the subsequent experiments.

VITAMIN D SUPPLEMENTATION DID NOT AFFECT PGE_2-INDUCED PAW EDEMA

An intraplantar injection of PGE_2 induces an intense local inflammatory response within the 1st hour after application of the phlogistic agent, with a maximum intensity at the 4th hour. Vitamin D supplementation (1 mg/kg) did not affect the evolution of the PGE_2-induced inflammatory response at any of the periods of evaluation (PGE_2 + Vit $D_{1.0}$: 49.7 ± 1.5 (p=0.99), 57.7 ± 1.7 (p=1.00), and 69.4 ± 1.3 (p=0.97), respectively) compared with the control group that did not receive vitamin D supplementation (PGE_2 group: 43.9 ± 3.4, 54.1 ± 3.5, and 63.6 ± 3.1, respectively). Treatment with NPX significantly reduced the development of edema at all periods of evaluation (PGE_2 + NPX group: 19.5 ± 1.4 (p<0.01), 31.4 ± 1.2 (p<0.01), and 41.8 ± 1.3 (p<0.01), respectively; Figure 2A).

VITAMIN D SUPPLEMENTATION DID NOT AFFECT DEXTRAN-INDUCED PAW EDEMA

Paw edema that is induced by dextran is characterized by a maximum intensity of edema within the 1st hour after application of the phlogistic agent, which occurs through the release of serotonin and histamine mediators.[14]. Supplementation with vitamin D (1 mg/kg) for 15 days did not affect the evolution of the inflammatory response (Dx + Vit $D_{1.0}$: 177.4 ± 8.8 (p=1.00), 224.2 ± 10.0 (p=0.99), 195.6 ± 14.7 (p=1.00), and 171.2 ± 11.1 (p=0.98) at 30, 60, 120, and 240 min, respectively) compared with the group that did not receive vitamin D supplementation (Dx group: 183.7 ± 9.0, 229.7 ± 5.9, 216.7 ± 3.2, and 198.2 ± 3.6, respectively). Treatment with Ci-Hep significantly reduced the evolution of edema at all periods of evaluation (Dx + Ci-Hep: 52.60 ± 10.28 (p<0.01), 54.60 ± 12.93 (p<0.01), 56.60 ± 15.61 (p<0.01), and 57.40 ± 14.20 (p<0.01), respectively; Figure 2B).

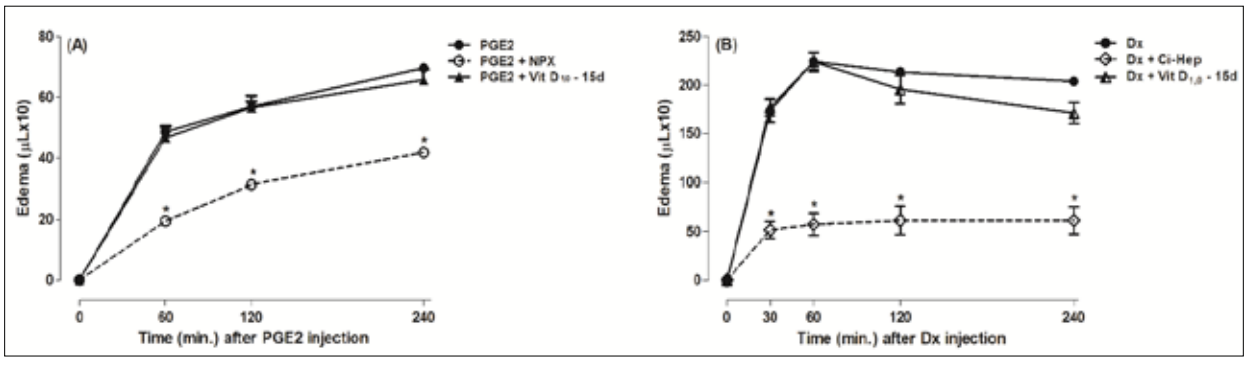

Figure 2. Effect of vitamin D (1 mg/kg) on the development of paw edema induced by an intradermal injection of prostaglandin E_2 (PGE_2) (**A**) and dextran (Dx) (**B**) in Wistar rats. The animals received oral vitamin D supplementation daily for 15 days before the injection of the phlogistic agent. PGE_2 and Dx groups, untreated animals that received PGE_2 and Dx. Naproxen (NPX; 3mg/kg) (**A**) and cyproheptadine (Ci-Hep; 10 mg/kg) (**B**) were used as reference drugs. The data are expressed as the mean paw volume ± SEM. $^{\#}p<0.05$ compared 1st, 2nd, and 4th hours after paw edema induced; $^{*}p<0.05$, compared with Cg group (repeated-measures analysis of variance followed by Tukey's test - Statistic 8.0 software - StatSoft®).

VITAMIN D SUPPLEMENTATION DID NOT AFFECT MYELOPEROXIDASE ACTIVITY

The intradermal injection of carrageenan significantly increased MPO activity (250% of increase). Supplementation with vitamin D (1 mg/kg) for 15 and 30 days or NPX did not significantly affect MPO activity (Figure 3).

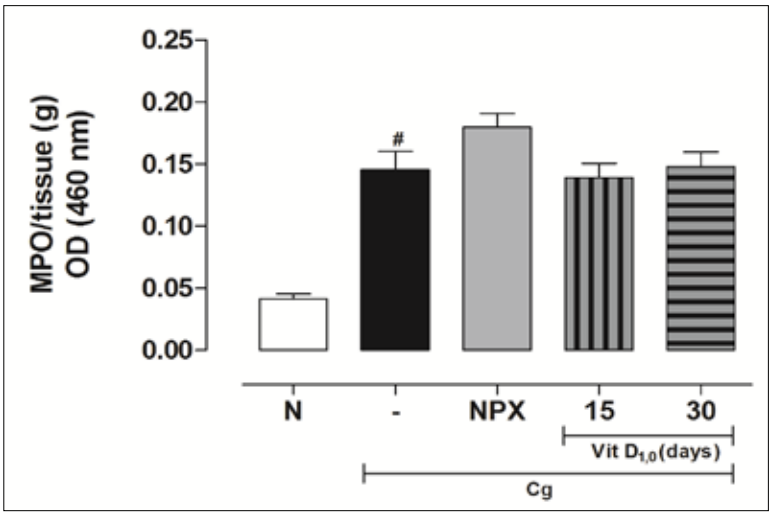

Figure 3. Effect of vitamin D supplementation (1 mg/kg) on myeloperoxidase activity in plantar tissue samples in Wistar rats. The animals received oral vitamin D supplementation for 15 or 30 days prior to the induction of paw edema. Cg, untreated control animals that received carrageenan. The positive control group was treated orally with naproxen (NPX 3 mg/kg). The data are expressed as the mean absorbance ± SEM (MPO/mg tissue) 4 h after the injection of carrageenan. $^{\#}p < 0.05$, compared with normal group (one-way ANOVA followed by Tukey's test).

VITAMIN D SUPPLEMENTATION DID NOT AFFECT NITRIC OXIDE CONCENTRATIONS

Supplementation with vitamin D (1 mg/kg) for 15 or 30 days did not significantly affect NO concentrations compared with the Cg group. Treatment with NPX significantly reduced NO concentrations ($p<0,05$) (Figure 4).

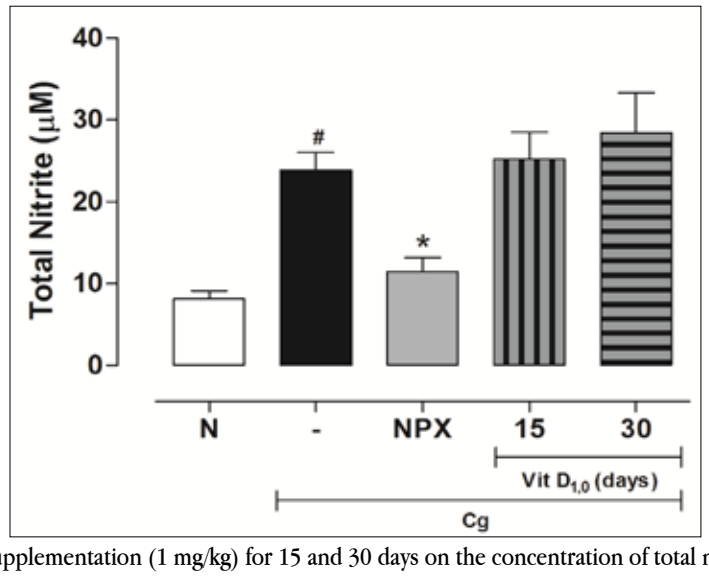

Figure 4. Effect of vitamin D supplementation (1 mg/kg) for 15 and 30 days on the concentration of total nitrite in plantar tissue in Wistar rats. The animals received oral supplementation with vitamin D for 15 or 30 days prior to the induction of paw edema. The normal group (N) and carrageenan group (Cg) received no treatment. The NPX group was treated orally with naproxen (NPX 3 mg/kg).. The data are expressed as the mean nitrite concentration ± SEM 4 h after the carrageenan injection. $^{\#}p < 0.05$, compared with normal group; $^{*}p < 0.05$, compared with carrageenan group (one-way ANOVA followed by Tukey's test).

VITAMIN D SUPPLEMENTATION REDUCED TNF-α CONCENTRATIONS

Daily supplementation with vitamin D for 15 and 30 days significantly reduced TNF-α concentrations (11% inhibition in both groups of animals; Figure 5).

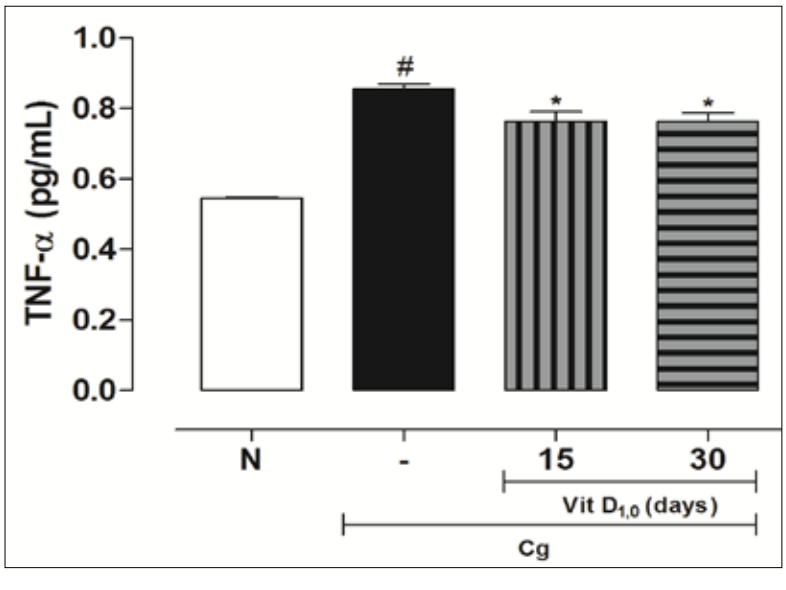

Figure 5. Determination of TNF-α levels in plantar tissue supernatants 4 h after the intraplantar injection of carrageenan. The animals received oral vitamin D supplementation (1 mg/kg) daily for 15 or 30 days. The normal group (N) and carrageenan group (Cg) received no treatment. The data are expressed as mean ± SEM. $^{*}p < 0.05$, compared with Cg group; $^{\#}p < 0.05$, compared with normal group (one-way ANOVA followed by Tukey's test).

DISCUSSION

The present study investigated the effect of vitamin D supplementation on the acute inflammatory response using different treatment protocols (single dose 1 h prior to edema induction or supplementation for 7, 15, or 30 days prior to edema induction). Vitamin D supplementation (1 mg/kg) for 15 and 30 days attenuates the development of the inflammatory response in model of carrageenan-induced paw edema.

The inflammatory response that is induced by carrageenan is characterized by acute inflammation, with formation of edema and intense leukocyte infiltration at the lesion site. This inflammatory response is associated with the production of inflammatory mediators that participate in both vascular and cellular phenomena. It is one of the most frequently used models for the study of acute inflammation, inflammatory pain, and the anti-inflammatory activity of different compounds[17].

Among the mediators that are involved in the inflammatory response that is induced by carrageenan, prostaglandin plays a key role in the development and maintenance of the process. However, in our study, vitamin D did not exert a direct inhibitory effect in the model of prostaglandin-induced edema. Therefore, we suggest that the observed antiinflammatory effect observed maybe related to actions of vitamin D on prostaglandin production or release. This hypothesis was supported by Moreno et al. (2005)[18], who showed that vitamin D inhibited the expression of cyclooxygenase 2 (COX-2) and increased prostaglandin catabolism.

We also found that the inhibitory effect of vitamin D on the inflammatory response did not appear to be related to histamine release. Vitamin D supplementation did not influence the development of dextran-induced edema, suggesting that vitamin D does not affect the release of these mediators from mast cell degranulation. According to Yip et al. (2014)[19], vitamin D causes a modest reduction of histamine release (*in vitro* and *in vivo*), and only a summation of effects of vitamin D on several different mediators results in an effective antiinflammatory response.

Supplementation with vitamin D (1 mg/kg) for 15 and 30 days significantly inhibited carrageenan-induced paw edema. We further investigated the possible mechanism of action that is involved in the observed antiedematogenic effect by evaluating leukocyte recruitment (i.e., MPO activity) and inflammatory mediators (NO and TNFα) in samples of plantar tissue. Myeloperoxidase is a proteolytic enzyme that is found in azurophil granules of neutrophils whose activity indirectly indicates the recruitment of leukocytes to sites of inflammation[15]. Nitric oxide plays an important role in the acute inflammatory process by promoting intense cellular recruitment and contributing to an increase in vascular permeability with the extravasation of fluids and proteins to inflamed tissue[16]. TNF-α is also an important proinflammatory mediator that plays a role in carrageenan-induced paw edema.

Although some authors have shown that vitamin D supplementation significantly reduced MPO activity and NO concentrations[20,21], we found no alterations of these markers of inflammation, suggesting that they are not involved in the antiinflammatory effect of vitamin D in this experimental model.

Nonetheless, we found that the inhibitory effect of vitamin D may be related to a reduction of TNF-α concentration. Previous studies found that vitamin D reduced the concentrations of this cytokine in both *in vivo* and *in vitro* models of inflammation [22,23]. Other studies have shown that antiinflammatory effects of vitamin D are related not only to its ability to reduce proinflammatory cytokine concentrations (e.g., interferon-γ, IL-6, IL-12, and TNF-α) but also to an increase in concentrations of antiinflammatory cytokines (e.g., IL-4 and IL-10)[22]; the latter were not evaluated in the present study. Vitamin D appears to act by inhibiting the activation of NF-κB, which is responsible for activating distinct pathways and mechanisms that promote the generation of proinflammatory cytokines, antiapoptotic factors, and enzymes that are responsible for the origin of proinflammatory mediators[8,24].

No treatment protocol has been defined in the literature for the use of vitamin D for the treatment of different diseases. Nevertheless, it is agreed that the doses used in these conditions are superior to those used for bone maintenance, which can vary from 400 to 800 IU/day[4]. Several authors have reported the beneficial effects

of vitamin D supplementation in various diseases, with doses that range from 400 IU/day to 40,000 IU/week[25,26]. Such effects and dosing corroborate the present results, in which vitamin D supplementation (8,000 IU/day) inhibited the development of the inflammatory response.

The daily administration of vitamin D at high doses does not appear to result in adverse effects. According to Garland et al. (2011)[27], daily doses up to 40,000 IU do not cause toxicity. Our results confirm these findings. Supplementation with 8,000 IU/day did not alter hepatic or renal function, the lipid profile, or glycemia in the supplemented animals (data not shown).

The animals that were supplemented with vitamin D presented less body weight gain compared with normal animals, and this response did not appear to be related to lower intake because no significant difference in food intake was observed (data not shown). These results validate the findings of Farhangi et al. (2017)[28], who also showed that vitamin D supplementation did not alter food intake. Consistent with Sun and Zemel (2008)[29] and Blum et al. (2008)[30], the prolonged use of vitamin D regulates lipolysis and the death of adipocytes and decreases fat mass, which may explain the present results. However, the mechanisms by which vitamin D interferes with these processes has not been elucidated and were not the objective of this study.

CONCLUSION

Altogether, the present results showed that vitamin D supplementation had an inhibitory effect on the development of the acute inflammatory response that was induced by carrageenan, and this effect was at least partially attributable to the reduction of TNF-α concentration. However, we cannot rule out other possible effects on the production/release of other inflammatory mediators. Our findings indicate that vitamin D supplementation may be a therapeutic alternative for controlling the inflammatory process.

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