Inflammation, Vol. 42, No. 5, October 2019 (© 2019)
DOI: 10.1007/s10753-019-01021-1

ORIGINAL ARTICLE

Co-Nanoencapsulation of Vitamin D_3 and Curcumin Regulates Inflammation and Purine Metabolism in a Model of Arthritis

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Abstract— We analyzed the effects of a nanoencapsulated association of curcumin and vitamin D_3 on purine metabolism enzymes in neutrophils, lymphocytes, and platelets in a model of adjuvant-induced arthritis (AIA) in rats. Following AIA induction, the animals were treated for 15 days with free and nanoencapsulated curcumin (4 mg/kg), nanocapsules of vitamin D_3 (VD3) (15.84 IU/day), a nanoencapsulated combination of curcumin and VD3, vehicle, or blank nanocapsules. The animals were euthanized, and blood was collected to evaluate the activities of E-NTPDase, adenosine deaminase (ADA), and myeloperoxidase (MPO), as well as reactive oxygen species (ROS) levels and biochemical parameters. Also, the liver and kidney were collected for histological analysis. The changes in the activities of purinergic enzymes indicated that inflammation was significantly reverted by vitamin D_3 and curcumin co-nanoencapsulation treatments in the arthritic rats. The reduction of inflammation was confirmed by the reduction in the signs and symptoms of AIA, as well as in MPO activity by all formulations. The treatments with nanocapsules reverted histological alterations in the kidney. Serum parameters were not altered by the induction or treatments. Our results suggest that co-nanoencapsulation of vitamin D_3 and curcumin is an efficient alternative adjuvant treatment for rheumatoid arthritis as it reverts the changes in the purine metabolism and reduces arthritis-associated inflammation.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10753-019-01021-1) contains supplementary material, which is available to authorized users.

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KEY WORDS: arthritis; purinergic system; inflammation; vitamin D_3; curcumin.

INTRODUCTION

Rheumatoid arthritis (RA) is an immune-mediated disease characterized by the activation and infiltration of inflammatory cells and proliferation of synovial tissue [1]. It can rapidly shift from a local reaction to a multisystem inflammation and may lead to an irreparable deterioration of the joints, causing debilitation and increasing the risk of mortality [2].

During the development of RA, interactions occur between local cells and the inflammatory cells of both acquired and innate immunities recruited of the site of injury. T and B cells, neutrophils, macrophages, and platelets release inflammatory mediators such as cytokines (IL-1 β, IL-6, tumor necrosis factor (TNF-α)), autoantibodies (rheumatoid factor (FR), and anti-citrullinated peptide antibodies (ACPAs) that amplify the damage [3, 4]. Therefore, the interactions between the immune cells generate an inflammatory process that contributes to the progress and maintenance of the signs and symptoms of the disease [5].

In the presence of endothelial damage, immune cells become activated and release ATP (adenosine triphosphate), a damage-associated molecular pattern (DAMP), resulting in proinflammatory signals. The purine metabolism comprises the hydrolysis of the adenine nucleotides ATP and ADP (adenosine diphosphate) by E-NTPDase (EC 3.6.1.5; CD39), AMP by E-5′-nucleotidase (EC 3.1.3.5; CD73), and the deamination of adenosine by E-ADA (ecto-adenosine deaminase; EC 3.5.4.4). These processes regulate not only the concentration of these metabolites but also their effects on the immune response within the extracellular environment [6]. Besides ATP, other purines also play biological roles as they interact with their specific purinoreceptors [7–9]. Adenosine, a metabolite of adenine nucleotide, is a limiting factor for inflammation which, in RA, prevents joint destruction and pain [10, 11]. The maintenance of RA depends on a continuous inflammatory process in which adenine nucleotides participate as signaling molecules [12]. The modulation of such a system may bring benefit to patients with chronic diseases such as RA.

A large number of serious adverse effects and limited efficacy coupled with the chronicity of current therapies for RA [13] has created the need for the development of new, safer, and more effective anti-arthritis therapies. In view of this, there has been a growing interest in the use of natural compounds like curcumin and the supplementation of vitamin D_3 as adjunctive therapy to prevent the development or delay manifestations of diseases such as RA [14–16]. The use of complementary therapies with natural compounds and substitution of metabolites has shown advantages in terms of safety and tolerability [17, 18]. In addition, patients with rheumatoid arthritis are more likely to accept complementary therapies to relieve the symptoms of RA [19].

Curcumin, a polyphenol derived from *Curcuma longa L.* (Zingiberaceae), is of particular importance for the treatment of diseases and inflammatory disorders, RA in particular [20–22]. However, the *in vivo* use of curcumin is limited by issues regarding absorption, distribution, bioavailability, and metabolism, in addition to its hydrolytic propensity and photochemical degradation [23, 24]. Unlike curcumin, vitamin D is an endogenous hormone which, in low levels, is related higher susceptibility to RA [25], thus vitamin D_3 supplementation becomes relevant in the context of prevention and possible reduction of damage related to arthritis [26, 27].

The use of nanotechnology is a convenient strategy to overcome the physicochemical and biopharmaceutical properties of curcumin [28]. In addition, the use of nanotechnology would optimize the non-musculoskeletal benefits of vitamin D_3, delivering this agent directly to inflammatory sites [29]. Besides, supplementation with nanoencapsulated vitamin D_3 is also useful since the daily needs of vitamin D are usually low and thus outweigh the risk of hypervitaminosis [30]. In previous studies by our group, we demonstrated the ability of nanoencapsulation to preserve efficacy with reduced concentrations of curcumin [31] and vitamin D_3 [30]. The use of bionanotechnology provided us and other researchers with improved anti-arthritic effects, significant dose reduction, and increased oral bioavailability and safety of both compounds [30, 32, 33].

Therefore, this study was designed to evaluate the *in vivo* effects of an innovative treatment for RA through the co-encapsulation of vitamin D_3 and curcumin in polymeric nanocapsules in a model of arthritis. These effects were evaluated through the purine metabolism on the surface of immune cells that are directly related to the immune response to RA and its pathogenesis [34]. In addition, histological and biochemical analyses, as well as MPO activity and the formation of ROS in adjuvant-induced

arthritis (AIA), were studied to support our novel contribution to the area.

MATERIAL AND METHODS

Animals

Adult female Wistar rats (150–250 g), heterogenic and conventional, originated from the UFSM Central Vivarium, were used. The animals were maintained under controlled conditions (22 ± 2 °C under a 12-h/12-h light/dark cycle) with food and water available *ad libitum*. The experimental protocol was approved by the Ethics Committee on the Use of Animal at the Federal University of Santa Maria (8937281117).

Reagents and Chemicals

The bovine serum albumin, Coomassie Brilliant Blue G, adenosine, and Complete Freund's Adjuvant (CFA) were bought from Sigma-Aldrich (St. Louis, MO, USA). The vitamin D_3 formulation used Addera D_3® oral solution (cholecalciferol-vitamin D_3—3300 IU), Mantecorp Farmasa®. Ficoll–Hypaque (Lymphoprep) was purchased from Nycomed Pharma (Oslo, Norway). The vehicle used was cooking corn oil. The curcumin was bought from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in the experiment were from analytical grade and highest purity.

Induction and Assessment of Arthritis and Treatment of Rats

Arthritis was induced by a single subcutaneous injection of 50 μL of a CFA suspension (Complete Freud's Adjuvant), which consists of killed and dried *Mycobacterium tuberculosis* (1.0 mg/mL), dissolved in liquid paraffin at a concentration of 0.6% or saline (used as a control), and administered into the right hind paw.

Tests were made to check the development of the inflammatory process 15 days after de-induction. The arthritis score, paw immersion latency (thermal hyperalgesia), and paw thickness (paw edema) were performed as described previously by our study group [30]. These tests were performed again 15 days after treatment, to analyze the effect of different treatments on signals of an inflammatory process associated with induced arthritis.

The arthritis score was used to assess the progression of arthritis, in which the signs of inflammation were observed and classified according to a scale [35, 36]. The paw immersion test evaluated the hypersensitivity to heat stimulation. In this test, the right hind paw of the animals was immersed in a water bath at 48 °C and the index of the thermal nociceptive threshold was given by measuring the amount of time from the beginning of the stimulus to the animal's response by withdrawing the paw [37]. The onset and development of edema were observed through the right hind paw thickness and were measured using a digital caliper [38].

Production and Characterization of the Nanocapsules Formulations

Lipid-core nanocapsules (NC, NC_{VD3}, NC_{CUR}, $NC_{VD3\text{-}CUR}$) were obtained by interfacial deposition of preformed polymer technique [39, 40]. The organic phase consisted of poly(ε-caprolactone) as a biodegradable polymer, sorbitan monostearate, TCM, or vitamin D_3 as oil, all dissolved in acetone. For those formulations containing curcumin, it was added to the organic phase together with all other components. The organic phase was injected into an aqueous phase containing polysorbate 80, a surfactant. Acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure to obtain 100 mL. Table 1 (Supplementary Material) shows the composition of the formulations, according to our previous reports [30, 31]. For the formulations containing CUR, its concentration was set to 0.50 mg/mL. All formulations were prepared in triplicates and kept protected from light at room temperature. The volume of vitamin D_3 added to the formulations NC_{VD3} and $NC_{VD3\text{-}CUR}$ corresponded to 528 UI, reaching a final concentration of 52.8 UI/mL in these formulations.

In the first characterization step, the particle size distribution of nanocapsules was evaluated by laser diffraction (Mastersizer® 2000, Malvern Instruments, Malvern, UK) to guarantee that the pharmaceutical composition was optimized to ensure the formation of particles exclusively at the nanoscale. Afterward, the particle size distribution and polydispersity index (PDI) of the formulations were measured by dynamic light scattering (Zetasizer Nano ZS®, Malvern Instruments, Malvern, UK) after a previous dilution of the formulations in ultrapure water. Zeta potential was evaluated by electrophoretic mobility (Zetasizer Nano ZS®, Malvern Instruments, UK). Curcumin concentration and its encapsulation efficiency were evaluated by liquid chromatography, as previously reported [40]. The analytical method was validated for these specific formulations, showing good linearity (1–20 μg/mL, $r = 0.9998$), precision (RSD of 1.79% and 2.16% for the intra- and

inter-day evaluation, respectively), and accuracy (recovery between 97.5 and 99.5%). Specificity was tested in the presence of all other components, showing that they do not interfere in the curcumin assay. Limits of quantification and detection were 0.28 and 0.08 μg/mL, respectively. The physicochemical characteristics of the formulations are exhibited in the Supplementary Material (Table S2).

Treatment

The treatments were initiated 15 days after induction and lasted for 15 days, following the model of previous studies [30]. All treatments were administered by oral gavage, and the vehicle used was corn oil.

The animals were divided into 14 groups: CN + VEHICLE (control), healthy untreated; CN + NC healthy treated with blank nanocapsules; CN + NC_{VD3} healthy treated with nanoencapsulated vitamin D_3; CN + NC_{CUR}, healthy treated with nanoencapsulated curcumin; CN + VD3-CUR healthy treated with a combination of vitamin D_3 and curcumin in the free form (not nanoencapsulated); CN + CUR, healthy treated with curcumin in the free form; CN + $NC_{VD3\text{-}CUR}$, healthy treated with a nanoencapsulated association of vitamin D_3 and curcumin; AIA + VEHICLE, untreated arthritic animals; AIA + NC, arthritic treated with blank nanocapsules; AIA + NC_{VD3}, arthritic treated with nanoencapsulated vitamin D_3; AIA + NC_{CUR}, arthritic animals treated with nanoencapsulated curcumin; AIA + VD3-CUR, arthritic treated with a combination of vitamin D_3 and curcumin in free form; AIA + CUR, arthritic treated with curcumin in the free form; and AIA + $NC_{VD3\text{-}CUR}$, arthritic treated with an association of vitamin D_3 and curcumin in the nanoencapsulated form.

NC_{VD3} groups received a previously prepared suspension (52.8 IU/mL VD3) which is equivalent to a dose of the 15.84 IU/day of the vitamin D_3. CUR groups received the formulation of 25 mg/kg of curcumin, dissolved in 0.3 mL of corn oil. NC_{CUR} groups received a formulation with nanocapsules of 4 mg/kg of curcumin. CN + VEHICLE and AIA + VEHICLE groups received corn oil and the NC groups received blank formulation, only with the components to the formation of nanocapsules.

Aiming to reduce the number of animals used in the experiment, the induction model and the doses, as well as the treatments, were done according to a previous study. Based on that, we did not include a group treated with free VD_3, which has shown results similar to the nanoencapsulated VD_3 although with higher doses in a previous study [25].

Experimental Procedure

The experimental design is demonstrated in Fig. 1. The assessment of arthritis (arthritis score, thermal hyperalgesia, and paw edema) was performed in three different time points: D0 (before induction), D15 (15 days after induction), and D30 (15 days after treatment).

At the end of the treatments, the animals were anesthetized by inhalation of isoflurane and submitted to euthanasia by exsanguination and blood was collected by cardiac puncture whole blood was used to separate the cells, serum, and plasma.

All animals were weighted on D7 (7 days after induction), D14 (14 days after induction), D21(21 days after induction) and D28 (28 days after induction).

Isolation of Lymphocytes and Neutrophils from Blood

Blood collected in a tube with 7.2 mg dipotassium EDTA (anticoagulant). The whole blood was layered in Ficoll–Histopaque according to Böyum [41]. After centrifugation and formation of different density layers, a lymphocyte-rich mononuclear cell layer and a polymorphonuclear cell layer were collected and washed with saline. The protocol was carried out according to manufacturer's instructions. The samples containing lymphocyte and neutrophil were used immediately for enzymatic assays.

Isolation of Platelets from Blood

For the isolation of platelets, platelet-rich plasma was separated from blood collected with anticoagulant sodium citrate 3.5% according to the method of Pilla et al. [42].

Evaluation of Cell Integrity

The integrity of cells was assayed by determining the lactate dehydrogenase (LDH) activity in intact and disrupted cells using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer).

Separation of Blood Serum and Plasma

The blood samples were collected in tubes without anticoagulant and gel serum separator, at room temperature, and were centrifuged at 1400×*g*/min for 15 min. The resultant serum samples were aliquoted in proper microtubes and frozen for further analyses.

Plasma was separated from blood collected with EDTA, followed by centrifugation at 1800×*g* for 10 min.

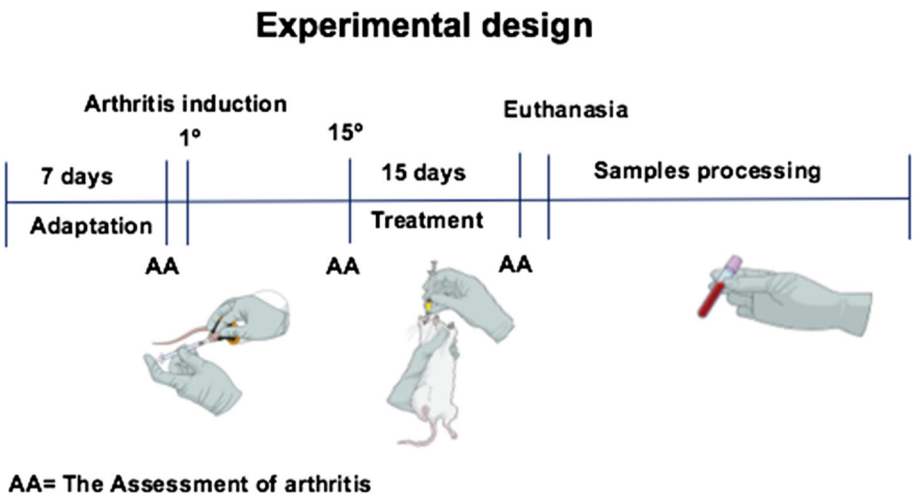

Fig. 1. Experimental design.

Protein Content

The protein quantification was measured by the Coomassie Blue method described by Bradford [43] using serum albumin as a standard.

NTPDase Activity Determination in Neutrophils

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 5 mM glucose, 50 mM tris-HCl buffer, and pH 7.4, at a final volume of 200 μL as describe by Lunkes et al. [44]. Twenty microliters of the enzyme preparation (8–12 μg of protein) was added to the reaction mixture and pre-incubation proceeded for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and the time of incubation was 60 min. The enzyme assay was stopped by the addition of 200 μL of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of Chan et al. [45] using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for non-enzymatic hydrolysis of nucleotides by adding enzyme preparation after TCA addition. All samples were run in triplicate. Enzyme-specific activities are reported as nanomole of Pi released per minute per milligram of protein.

Adenosine Deaminase Activity Determination

The method previously described by Giusti and Galanti [46] was used to measure E-ADA activity in lymphocytes, neutrophils, and platelets, and ADA in serum, based on the direct quantification of ammonia produced during the deamination of adenosine by ADA.

Twenty-five (25) microliters of cells or serum was added to 21 mM/L of adenosine, pH 6.5, and incubated for 90 min at 37 °C. By adding 106.2 mM phenol and 167.8 mM sodium nitroprusside and hypochlorite solution, the reaction was stopped. Seventy-five (75) micromoles of ammonium sulfate was used as a standard. The protein content was adjusted for lymphocytes (0.1–0.2 mg/mL), neutrophils (0.2–0.3 mg/mL), and platelets (0.4–0.6 mg/mL). All samples were run in triplicate and E-ADA activity was expressed in micrometer of NH_3 per minute per milligram of protein and serum ADA activity was expressed in unit per liter. One unit (1 U) equals the amount of enzyme needed to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

Myeloperoxidase (MPO) enzyme activity in plasma

MPO activity in plasma was analyzed spectrophotometrically by a modified peroxidase-coupled assay systeminvolving phenol, 4-aminoantipyrine (AAP) and H_2O_2 as previously described by Metcalf et al. [47]. Briefly, 390μL of 2.5 mM AAP, 20 mM of phenol, and 30μL of the sample will be placed in each tube, followed by 450 μL of 1.7 mM H_2O_2. In the presence of H_2O_2 as the oxidizing agent, the oxidative coupling of phenol and AAP iscatalyzed

by MPO to give a colored product, quinoneimine,its absorbance was read at 492 nm. The results were expressed in micromolar of the quinoneimine produced in 30 min.

Reactive Oxygen Species in Plasma

ROS was measured using the 2′-7′dichlorofluorescein fluorescence assay according to Myhre et al. [48]. The aliquots of plasma (50 μL) are added to a medium containing Tris-HCl buffer (0.01 mM, pH 7.4) and DCFH DA 2′-7′-dichlorofluorescein-diacetate (1 mM). After the addition of DCFH-DA, the medium is incubated in the dark for 1 h until fluorescence measurement (excitation at 488 nm and emission at 525 nm). Dichloro-oxidized fluorescein is determined using a standard curve of oxidized dichlorofluorescein and the results are expressed as DCFH-DA fluorescence.

Histological Analyses

The liver and kidney were dissected for histopathological analysis. All samples were fixed in 10% buffered formalin and were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and analyzed by a pathologist under optical microscopy (Olympus Bx40) using the program Univision rel.48.

Biochemical Parameters

The biochemical analyses of serum samples were performed using a semi-automatic chemistry analyzer (Bioplus, BIO-2000), using commercial kits (Bioclin/Quibasa, Minas Gerais, Brazil), following manufacturer's recommendations. The biochemical parameters measured were creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST).

Statistical Analysis

Statistical analysis was performed by Graphpad Prism 7.0 software. First, the data were tested for normality of Kolmogorov–Smirnov and then analyzed with two-way analysis of variance (ANOVA) between groups, followed by the *post hoc* test of Tukey. The tests of assessment of arthritis were evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni's test. The results were exhibited as mean ± SEM, and a value of $p < 0.05$ was considered statistically significant.

RESULTS

Evaluation of Arthritis Induction and the Anti-Inflammatory Effects of Treatments

To confirm the success of the induction of inflammatory arthritis by CFA, we evaluated three signals of chronic inflammation: arthritis score (Fig. 2a), paw thermal latency (Fig. 2b), and paw edema (Fig. 2c). We also investigated the possible anti-inflammatory effects of treatments in chronic inflammation.

Fifteen days after the CFA injection, the arthritis score increased significantly (Fig. 2a), the paw thermal latency was considerably decreased (indicating the development of thermal hyperalgesia) (Fig. 2b), and the paw thickness showed a large increase (paw edema) (Fig. 2c) in all the AIA groups, indicating a successful induction of arthritis.

Once chronic inflammation was established, all free-form or nanocapsule treatments were successful in reducing clinical symptoms after 15 days of dosing; however, curcumin and vitamin D_3 co-nanoencapsulation treatments achieved the most significant results. The results showed a reduction in the arthritis scores (Fig. 1a), thermal hyperalgesia (Fig. 2b)(by the increased in latency time), and paw edema (Fig. 2c). At the same time, treatment with NC_{VD3} only reduced thermal hyperalgesia (Fig. 2b) and paw edema (Fig. 2c) 32.5 ± 10.6 and 42.9 ± 10.5, respectively. The injection of CFA did not induce notably behavioral alterations.

Weight Gain

The analysis of animal weight gain after arthritis induction is shown in Fig. 4. All animals with induced arthritis showed an intense and profound reduction in total weight, as demonstrated, only the arthritic animals sustained weight loss after the second week of evaluation. The AIA + VEHICLE animals maintained a reduction in body weight throughout the experiment. In contrast, treatments with VD3-CUR, CUR, N_{CUR}, and $NC_{VD3\text{-}CUR}$, after showing a reduction in the first 7 days after induction, returned to gain weight normally. The groups of non-induced animals did not show changes in weight gain, as did the arthritic animals in the NC group (data not shown).

E-NTPDase Activity in Neutrophils

The activity of E-NTPDase degrading ATP in neutrophils is shown in Fig. 5a. The AIA vehicle group (134.57 ± 10.3, $p < 0.05$, $n = 5$) showed a significant reduction in the catalytic activity in relation to the vehicle control group (CN-VEHICLE) (545.79 ± 67.7). The groups VD3-CUR

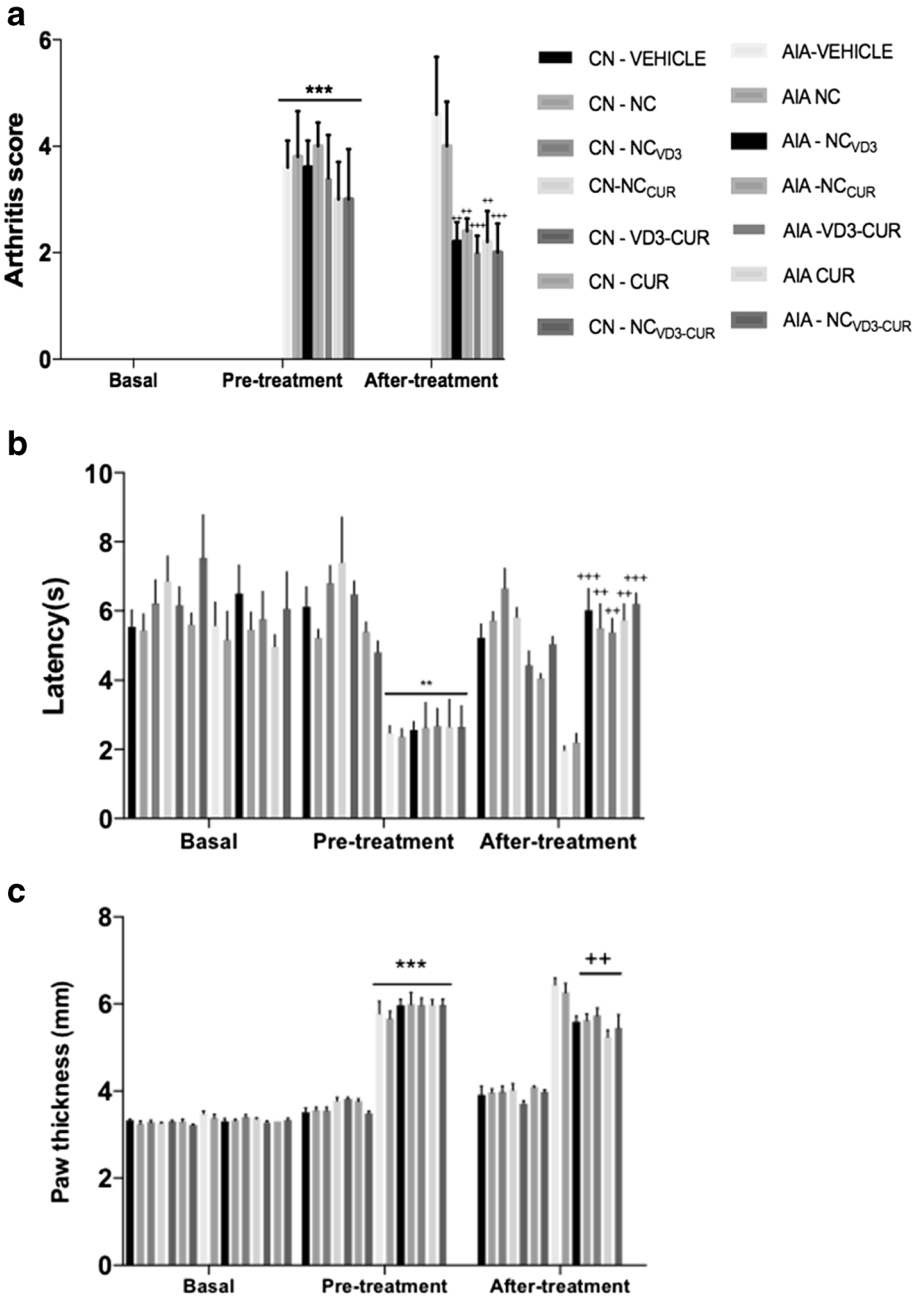

Fig. 2. Effect of the treatments (free solution and nanoencapsulated) on clinical inflammatory changes induced by intraplantar CFA. The evaluation on the arthritis score increase (**a**), thermal hyperalgesia (**b**), and edema formation (**c**) were performed in rats induced by arthritis (AIA) and control rats treated for 15 days with corn oil (VEHICLE), blank nanocapsules (NC), vitamin D_3 nanocapsules (NC_{VD3}), curcumin nanocapsules (NC_{CUR}), association of vitamin D_3 and curcumin in free form (VD3-CUR), curcumin (CUR) and association of vitamin D_3, and curcumin in nanocapsules ($NC_{VD3\text{-}CUR}$). The results were analyzed using two-way ANOVA followed by *post hoc* of Bonferroni. The results were expressed as the mean ± SEM. Asterisk (*) indicates that the value is significantly different from the control group. Plus sign (+) indicates that the value is significantly different from the AIA vehicle group. *($p < 0.05$, $n = 5$), **($p < 0.01$, $n = 5$), ***($p < 0.001$, $n = 5$), $^{+}$($p < 0.05$, $n = 5$), $^{++}$($p < 0.01$, $n = 5$), $^{+++}$($p < 0.001$, $n = 5$).

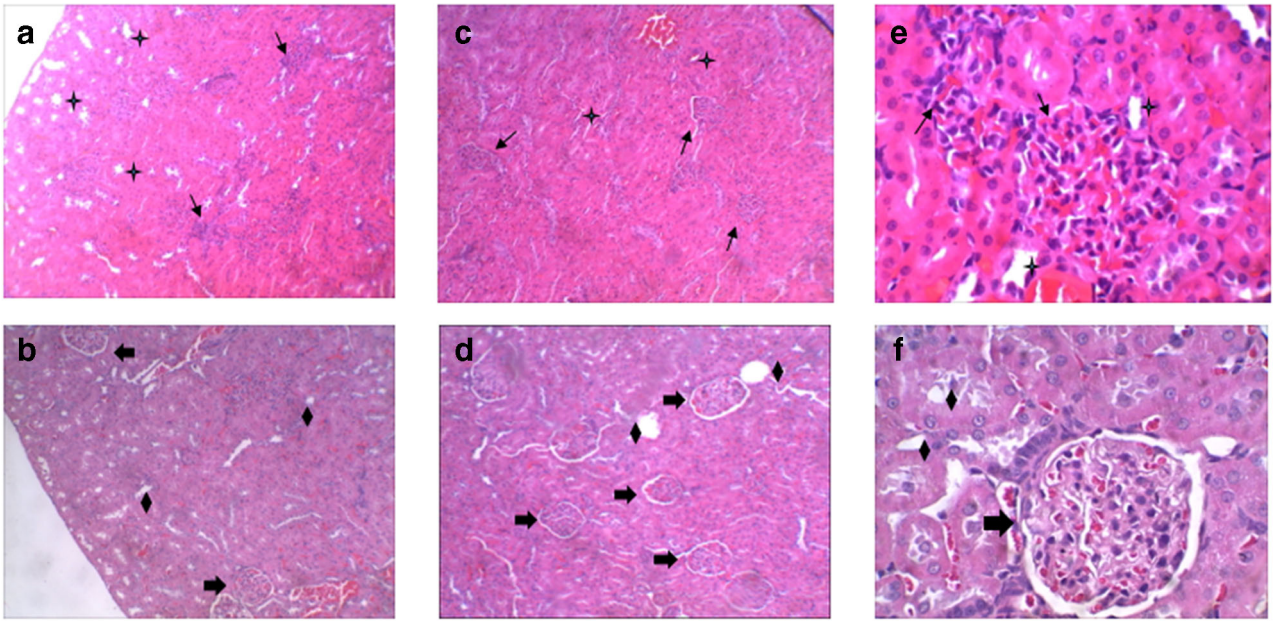

Fig. 3. Histological photomicrographs using hematoxylin and eosin staining of the kidney (**a**–**d** × 100; **e** and **f** × 400) ($n = 5$); black arrows indicate disorganized glomerulus; stars indicate compaction of the renal tubules; arrow thick indicates glomerulus well defined, and rhombus indicates normal-sized renal tubules. **a** AIA vehicle group, **b** AIA + NC_{VD3}, **c** AIA + NC, **d** AIA + NC_{CUR}, **e** AIA vehicle group, and **f** AIA + $NC_{VD3\text{-}CUR}$.

(560.77 ± 42.44, $p < 0.05$, $n = 5$) and $NC_{VD3\text{-}CUR}$ (592.93 ± 32.0, $p < 0.05$, $n = 5$) showed an elevation in ATP degradation in animals with induced arthritis. The AIA NC group (208.30 ± 38.5) had similar results to the AIA vehicle group. In relation to the groups with induced arthritis, the CUR (501.81 ± 122.75), NC_{CUR} (485.28 ± 122.75), and NC_{VD3} (422.8 ± 56.9) groups did not show a significant increase in the activity of this enzyme when compared with AIA vehicle group.

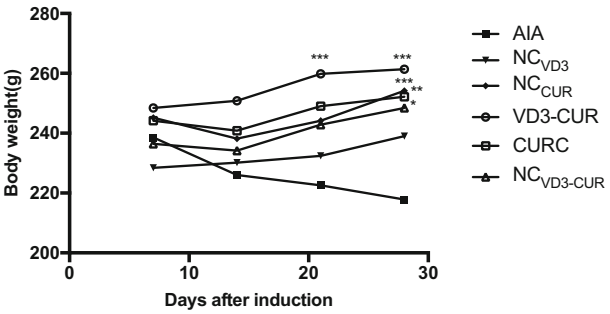

Fig. 4. Effect of the treatments (free solution and nanoencapsulated) on body weight of AIA rats (except NC group). Changes of body weight in arthritis-induced rats (AIA) treated for 15 days vitamin D_3 nanocapsules (NC_{VD3}), curcumin nanocapsules (NC_{CUR}), the association of vitamin D_3, and curcumin in free form (VD3-CUR), curcumin (CUR) and association of vitamin D_3, and curcumin in nanocapsules ($NC_{VD3\text{-}CUR}$). The results were expressed as the mean ± SEM. Asterisk (*) indicates that the value is significantly different from the AIA group *($p < 0.05$, $n = 5$), **($p < 0.01$, $n = 5$), ***($p < 0.001$, $n = 5$).

Figure 5b shown the ADP hydrolysis by E-NTPDase. The breakdown of ADP is similar to the ATP hydrolysis seen in the AIA vehicle group (147.55 ± 16.8, $p < 0.05$, $n = 5$) and AIA NC (167.65 ± 25.2, $p < 0.05$, $n = 5$), which exhibited diminished enzymatic activity when compared with the vehicle control group (505.96 ± 21.2). The AIA animals in VD3-CUR group (496.58 ± 46.0, $p < 0.05$, $n = 5$) and $NC_{VD3\text{-}CUR}$ group (582.45 ± 27.5, $p < 0.01$, $n = 5$) demonstrated an increased catalytic activity in relation to AIA vehicle group. The results of AIA NC_{CUR} (465.75 ± 62.3), CUR (467.76 ± 111.3), and NC_{VD3} groups (472.66 ± 48.9) were not significantly different.

E-ADA Activity in Neutrophils

The degradation of adenosine by E-ADA of neutrophils is shown in Fig. 6a. The arthritis-induced group (229.9 ± 19.0, $p < 0.05$, $n = 5$) showed a significant increase in the catalytic activity of E-ADA in relation to the vehicle control group (81.24 ± 18.7). The treatments with NC_{VD3} (77.13 ± 17.74, $p < 0.05$, $n = 5$), VD3-CUR (77.88 ± 17.23, $p < 0.05$, $n = 5$), and $NC_{VD3\text{-}CUR}$ (78.93 ± 13.76, $p < 0.05$, $n = 5$) significantly reverted the increase of E-ADA activity in

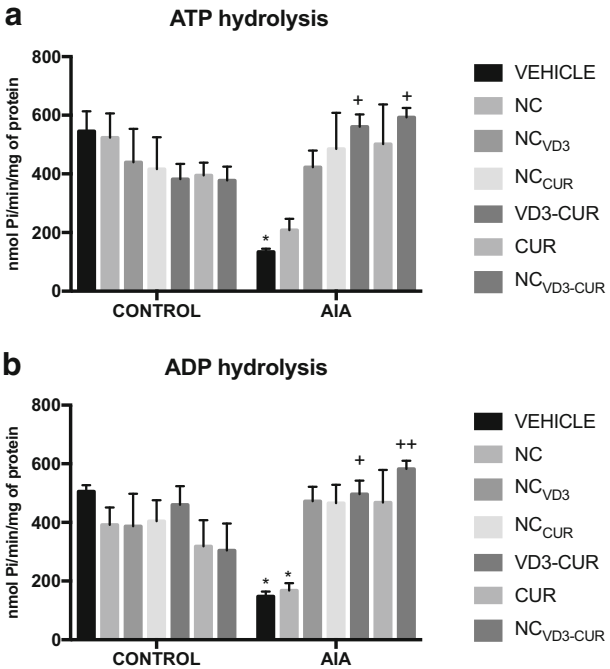

Fig. 5. E-NTPDase activity in ATP and ADP hydrolysis in neutrophils of arthritis-induced rats (AIA) and control rats treated for 15 days with corn oil (VEHICLE), blank nanocapsules (NC), vitamin D_3 nanocapsules (NC_{VD3}), curcumin nanocapsules (NC_{CUR}), association of vitamin D_3 and curcumin in free form (VD3-CUR), curcumin (CUR) and association of vitamin D_3 and curcumin in nanocapsules ($NC\text{-}_{VD3\text{-}CUR}$). Enzyme activities are reported as nmol Pi/min/mg of protein. The results were analyzed using two-way ANOVA followed by *post hoc* of Tukey. The results were expressed as the mean ± SEM. Asterisk (*) indicates that the value is significantly different from the control group. Plus sign (+) indicates that the value is significantly different from the AIA vehicle group.*($p < 0.05$, $n = 5$), $^{+}$($p < 0.05$, $n = 5$), $^{++}$($p < 0.01$, $n = 5$).

animals with induced arthritis. The AIA NC group had results similar to the vehicle group, and the CUR (123.22 ± 40.75) and NC_{CUR} groups (130.78 ± 27.18) did not show a significantly decreased activity of this enzyme.

E-ADA Activity in Lymphocytes

The E-ADA activity in lymphocytes is demonstrated in Fig. 6 b. The adenosine hydrolysis was significantly lower in the AIA + VEHICLE group (91.8 ± 12.74, $p < 0.01$, $n = 5$) and AIA NC (86.67 ± 21.66, $p < 0.01$, $n = 5$) when compared with control vehicle (257.44 ± 30.82); the NC_{VD3} control (120.27 ± 9.60) and $NC_{VD3\text{-}CUR}$ control groups (178.94 ± 29.16) had similar effects in enzymatic activity in relation to vehicle control group. After 15 days of treatment, the AIA NC_{VD3} (222.15 ± 22.6, $p < 0.05$, $n = 5$), AIA VD3-CUR (232.50 ± 22.28, $p < 0.05$, $n = 5$), and AIA $NC_{VD3\text{-}CUR}$ groups (230.06 ± 25.21, $p < 0.05$, $n = 5$) showed a significantly increased in E-ADA activity compared with the AIA vehicle group. The groups treated with CUR and NC_{CURC} did not show a reversion of E-ADA activity.

E-ADA Activity in Platelets

Figure 6c shows E-ADA activity in platelets. The degradation of adenosine was significantly higher in AIA (7.28 ± 0.45 $p < 0.01$, $n = 5$) and AIA + NC (6.44 ± 0.76 $p < 0.05$, $n = 5$) than in the CN + VEHICLE group (2.64 ± 0.68). All control groups demonstrated similar adenosine deamination. Only VD3-CUR (3.40 ± 0.36, $p < 0.05$, $n = 5$) and $NC_{VD3\text{-}CUR}$ (3.30 ± 0.28, $p < 0.05$, $n = 5$) were capable to significantly revert the increase in ADA activity, showing values for similar basal.

ADA Activity in Serum

The measurement of ADA in serum (Fig. 6d) showed a significant increase in the activity of ADA in the groups of rats with induced arthritis, AIA + VEHICLE (13.42 ± 0.61) and AIA + NC (13.02 ± 1.2) compared to the CN + VEHICLE group (6.54 ± 0.73). All treatments were significantly efficient in revert the activity of ADA in serum.

Myeloperoxidase Enzyme Activity in Plasma

The activity of the MPO is shown in Fig. 7. The AIA vehicle group (3.87 ± 0.24) showed a significant increase compared to the CN + VEHICLE group (2.20 ± 0.46). Regarding all the treatments, the NC_{VD3} (2.59 ± 0.36), NC_{CUR} (2.12 ± 0.17), VD3-CUR (1.63 ± 0.12), CURC (1.83 ± 0.19), and $NC_{VD3\text{-}CUR}$ (1.7 ± 0.17) has shown a significant reduction in the MPO activity when compared AIA vehicle group.

Reactive Oxygen Species in Plasma

The results of the reactive oxygen species levels in plasma are demonstrated in Fig. 8. The AIA groups and the control groups treated or untreated, showed no difference in ROS levels.

Histological Analyses

No relevant histopathological changes were observed in the hepatic analyses of the animals, healthy or arthritic, untreated or treated with nanocapsules, or with a free formulation. All liver samples were well preserved with a normal hepatic capsule, liver space, and liver parenchyma (data not shown). Furthermore, in any of the nanocapsule-

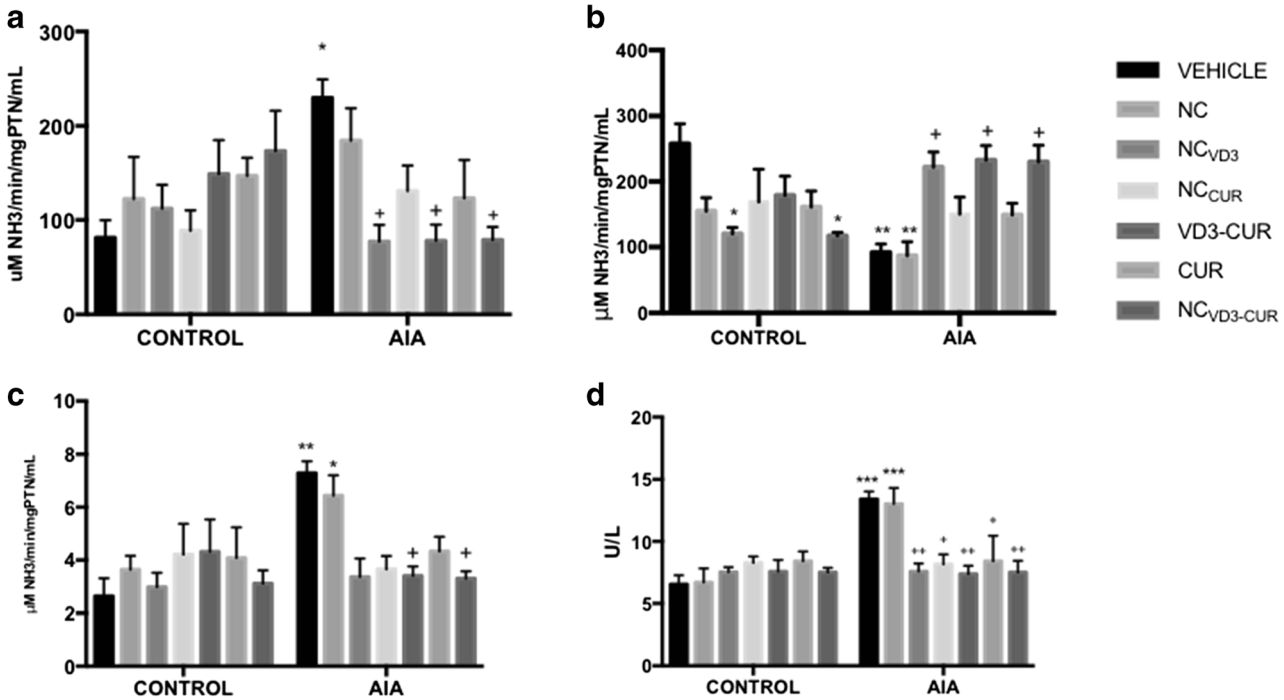

Fig. 6. Adenosine deamination in neutrophils (**a**), lymphocytes (**b**), platelets (**c**), and serum (**d**) of arthritis-induced rats (AIA) and control rats treated for 15 days with corn oil (VEHICLE), blank nanocapsules (NC), vitamin D_3 nanocapsules (NC_{VD3}), curcumin nanocapsules (NC_{CUR}), association of vitamin D_3 and curcumin in free form (VD3-CUR), curcumin (CUR) and association of vitamin D_3, and curcumin in nanocapsules ($NC_{VD3\text{-}CUR}$). Enzymatic activities are reported as micrometer of NH_3 per minute per milligram of protein in cells and unit per liter in serum. The results were analyzed using two-way ANOVA followed by *post hoc* of Tukey. The results were expressed as the mean ± SEM. Asterisk (*) indicates that the value is significantly different from the control group. Plus sign (+) indicates that the value is significantly different from the AIA vehicle group. *($p < 0.05$, $n = 5$), **($p < 0.01$, $n = 5$), ***($p < 0.001$, $n = 5$), $^{+}$($p < 0.05$, $n = 5$), $^{++}$($p < 0.01$, $n = 5$).

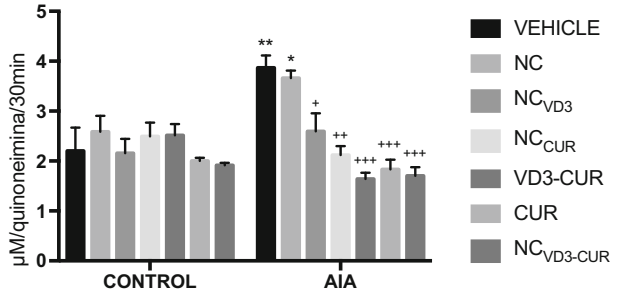

Fig. 7. Myeloperoxidase activity in plasma of arthritis-induced rats (AIA) and control rats treated for 15 days with corn oil (VEHICLE), nanocapsules blank (NC), nanocapsules with vitamin D_3 (NC_{VD3}), nanocapsules with curcumin (NC_{CUR}), association of vitamin D_3 and curcumin in free form (VD3-CUR), curcumin (CUR) and association of vitamin D_3, and curcumin in nanocapsules ($NC_{VD3\text{-}CUR}$). Enzyme activities are reported as (µM/quinoneimine/30 min). The results were analyzed using two-way ANOVA followed by *post hoc* of Tukey. The results were expressed as the mean ± SEM. Asterisk (*) indicates that the value is significantly different from the control vehicle group. Plus sign (+) indicates that the value is significantly different from the AIA vehicle group. *($p < 0.05$, $n = 5$), **($p < 0.01$, $n = 5$), $^{+}$($p < 0.05$, $n = 5$), $^{++}$($p < 0.01$, $n = 5$), $^{+++}$($p < 0.001$, $n = 5$).

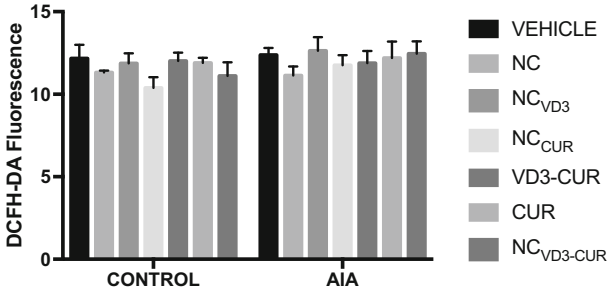

Fig. 8. Reactive oxygen species in plasma of arthritis-induced rats (AIA) and control rats treated for 15 days with corn oil (VEHICLE), nanocapsules blank (NC), nanocapsules with vitamin D_3 (NC_{VD3}), nanocapsules with curcumin (NC_{CUR}), association of vitamin D_3 and curcumin in free form (VD3-CUR), curcumin (CUR) and association of vitamin D_3, and curcumin in nanocapsules ($NC_{VD3\text{-}CUR}$). Dosage is reported as (DCFH-DA fluorescence). The results were analyzed using two-way ANOVA followed by *post hoc* of Tukey. The results were expressed as the mean ± SEM.

treated animal samples, no granulomatous body formation was found.

In relation to the histopathological analysis of the kidneys (Fig. 3), vehicle + AIA animals showed a certain compression of the renal tubules, greater vascularization, and disorganization of the glomeruli in relation to the control animals. Animals that were not induced or those with induced arthritis and treated with nanocapsules of curcumin and vitamin D_3 or free forms did not present histopathological alterations.

Biochemical Parameters

The biochemical parameters, ALT, AST, and creatinine tested in the serum of all groups did not present any difference of values (data not shown).

DISCUSSION

This study investigated a novel formulation of nanocapsules, comprising vitamin D_3 and curcumin co-encapsulated, on the inflammatory process of AIA in neutrophils, lymphocytes, and platelets through purine metabolism as well as histological, biochemical, and oxidative stress parameters.

Fifteen days post-induction of arthritis with CFA, the animals showed significantly increased paw edema and arthritis score measured by paw swelling, coloration, and claw position. Also, a reduction in the latency time in arthritic animals was observed, signaling that the animals became more sensitive to pain and thermal hyperalgesia. Together, these observations indicate the success of the induction of arthritis by the establishment of an inflammatory process (swelling, pain, color). All these clinical symptoms were reverted by the treatments.

Our data suggest the incapacity and severe pain felt by the arthritic animals may have compromised their food intake leading to weight loss. However, the treatments were successful in reducing these effects and causing the animals to maintain their normal eating regimen and expected weight gain. Although all treatments had a positive effect, the co-nanoencapsulation had the most prominent effect in reducing the signs and symptoms of the disease with a noteworthy dose reduction.

As fundamental cells to the onset and advancement of RA in humans [34, 49–51] and mice [52, 53] neutrophils have been gaining increased attention. Neutrophils release cytotoxic products and promotes nucleotide signaling through the release of ATP by neutrophil-secreting vesicles as a pro-inflammatory signal molecule, contributing to the pathogenesis and maintenance of RA [34, 49–51, 54]. The release of ATP by neutrophils results in autocrine purinergic signaling that results in increased chemotaxis and activation of these cells, causing damage in the sites of inflammation [55].

E-NTPDase degrades ATP [56, 57], consequently reducing the migration of other neutrophils, as well as competing for the directed actions of ATP on receptors such as P2X7, which is well described as pro-arthritogenic [58]. Our data show a reduction in the activity of E-NTPDase, degrading both ATP and ADP, in untreated animals with induced arthritis sustained an inflammatory status. A possible synergistic anti-inflammatory effect can be described in relation to the association of VD_3 and curcumin in the neutrophil E-NTPDase activity. The increased activity of this enzyme degrading ATP is related to negative modulation of neutrophil function with regard to vitamin D_3 [59] and a decrease in infiltration and activation by curcumin [60, 61].

The formation of adenosine by the hydrolysis of extracellular nucleotides acts as a mechanism that counteracts the inflammatory effects of the disease. However, the action of the E-ADA controls and finalizes the anti-inflammatory actions of the adenosine [62]. In general, ADA activity is increased in inflammatory diseases, such as rheumatoid arthritis [63]. In our study, animals with induced arthritis had significantly higher adenosine degradation by E-ADA than healthy animals, maintaining an inflammatory profile. Our treatments with free or nanoencapsulated association were effective in reducing the activity of this enzyme, as well as the treatment with NC_{VD3}.

Among the proposed synergistic effects, binding of 1α, 25 (OH)2D3 (VD_3) to vitamin D receptors (VDRs) in neutrophils causes a reduction in the release of IL-1β [62], a proinflammatory cytokine with a well-described role in the pathogenesis of arthritis. IL-1β, together with the reduction of matrix metalloproteinase expression and the induction of apoptosis of synovial fibroblasts [20], may reduce the activation of neutrophils and other cells leading to a reduction in ATP release [64] and consequent activation of the purinergic cascade until the degradation of adenosine.

ADA activity was verified in different cellular subtypes to profile this enzyme in RA. Unlike in neutrophils, the activity of E-ADA degradation in lymphocytes has been shown to be reduced in animals with arthritis [30]. On previous studies, our group found a reduction of the activity of E-ADA in lymphocytes of patients with RA [65]

and in an animal model of arthritis [30]. The kinetic difference observed in this cellular subtype can be explained by the body's attempt to counterbalance the inhibitory effects of E-ADA on adenosine, allowing for the maintenance of sufficient levels of this nucleoside. The actions of adenosine on T cells are mainly due to its high-affinity binding to the A2a receptor [66] which mediates intracellular signals that redirect a proinflammatory response to an immunosuppressive phenotype [67]. The treatments with the association (free and nanoencapsulated) and nanoencapsulated vitamin D_3 were able to revert the activity of this ectoenzyme into values similar to the control.

In fact, vitamin D_3 inhibits T lymphocyte proliferation and alters immunomodulatory responses by promoting a shift in the cellular phenotype from Th1 to Th2 [68, 69]. Curcumin appears to exert anti-proliferative effects on T cells [70], as well as inhibitory of antigen presentation [71]. Thus, their combined effects combat inflammation by lessening the activation and proliferation of T cells, thereby preventing the changes in lymphocyte E-ADA activity of arthritic rats.

Platelets play an important part in the joint lesion and systemic damage in arthritis [72]. Platelets from RA patients are hyperresponsive [73] and bind to other leukocytes, facilitating their activation and to adhesion to the endothelium [74], giving impetus to local inflammation. Corroborating to that, we found that adenosine deamination was elevated in platelets, likewise in neutrophils, after the induction of arthritis. These results, and those found in other studies with RA patients [63, 75], suggest a decrease in circulating adenosine levels and consequent poor prognosis [63], strengthening the idea that a set of interactions between these cells may be occurring. Our results showed that both the free association (VD3-CUR) and the nanoencapsulated association ($NC_{VD3\text{-}CUR}$) in AIA decreased E-ADA activity in platelets of arthritic rats.

Serum ADA, in the context of RA, is well described as a diagnostic marker and disease severity [76, 77]. As previously seen in patients [78], arthritic animals presented elevated ADA activity in serum in our study; however, treatments in both free and nanoencapsulated forms were able to decrease this activity. The generation of adenosine by the ectonucleotidases and the control of the ADA activity act as a mechanism to preserve sufficient levels of this nucleoside, favoring the control of the inflammatory state related to arthritis can be suggested. It is worth mentioning that the treatments in association showed a more pronounced decrease in ADA activity in serum, once again, indicating a possible synergistic effect of the association in the modulation of the purinergic system during the inflammatory process.

The increase in MPO observed in arthritic animals had already been reported in RA patients, indicates inflammation and neutrophil activation, may play an important role in the innate immune activation, and may also take part in the pathogenesis and severity of the disease [79, 80]. However, the decrease in MPO generation by treatments with curcumin and vitamin D_3 in nanocapsules indicate an important inhibitory effect of this inflammatory pathway [81]. Following the induction of arthritis, no increase in the production of ROS in serum was observed. Also, ROS levels remained unchanged after the treatments, proposing an independent relationship between ROS levels in serum with the presence of disease or with MPO levels in plasma. Stamp et al. [79] revealed that plasma MPO directly correlates with RA disease activity, as well an increase in both MPO levels and oxidative stress in synovial fluid. Although MPO catalyzes reactions responsible for the formation of ROS, it may not be responsible for raising it at the serological level, but locally in the affected tissue.

The histological changes found in the kidney of untreated arthritic rats may indicate the development of membranous nephropathy, glomerulitis, vasculitis, or secondary amyloidosis. However, renal involvement in arthritis is rare and there are few histopathological patterns for comparison [82, 83]. Nevertheless, these changes have been repaired by co-nanoencapsulation treatment. The administration of the nanocapsules *per se* did not demonstrate apparent histological alterations in the hepatic and renal structures. These data confirm that the dose used in our study is safe compared to a toxicity study performed by Bulcão et al. [84] who administered nanocapsules by the intraperitoneal route.

The association of vitamin D_3 and curcumin had a protective effect by modulating the purine metabolism in cells involved in the onset and maintenance of RA, in order to promote a shift to an anti-inflammatory profile. On a clinical perspective, this modulation was reflected in a significant reduction of the symptoms of arthritis. The co-nanoencapsulation of curcumin and vitamin D_3 has offered an effective and safe alternative capable to overcome the instability barriers presented by the compounds, in addition to an opportunity to increase adherence with a once daily dose.

CONCLUSION

To the best of our knowledge, this is the first work to produce and evaluate the co-nanoencapsulation of vitamin D_3 and curcumin in an arthritis model. We demonstrated a positive effect in modulation of purinergic metabolism

against the inflammation, showing also that the co-nanoencapsulation promoted the most significant results. The effectiveness, dose reduction, and safety found in our study indicate that the association of vitamin D_3 with curcumin nanoencapsulation is a good alternative to be further explored as an adjuvant in the treatment of this disease.

ACKNOWLEDGMENTS

The authors would like to thank Professor Volnei Teixeira at the Unijuí for his support in the interpretation of histology.

FUNDING INFORMATION

This study was partly financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)—Financial Code 001 (PROEX number: 88882.182182/2018-01).

COMPLIANCE WITH ETHICAL STANDARDS

The experimental protocol was approved by the Ethics Committee on the Use of Animal at the Federal University of Santa Maria (8937281117).

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