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Alpha-tocopherol-loaded polycaprolactone nanoparticles improve the inflammation and systemic oxidative stress of arthritic rats

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ABSTRACT

Background and aim: The present study investigated the effects of orally administered α-tocopherol-loaded polycaprolactone nanoparticles on the articular inflammation and systemic oxidative status of middle-aged *Holtzman* rats with Freund's adjuvant-induced polyarthritis, a model for rheumatoid arthritis. Intraperitoneally administered free α-tocopherol provided the reference for comparison.
Experimental procedure: Two protocols of treatment were followed: intraperitoneal administration of free α-tocopherol (100 mg/kg i.p.) or oral administration of free and nanoencapsulated α-tocopherol (100 mg/kg p.o.). Animals were treated during 18 days after arthritis induction.
Results: Free (i.p.) and encapsulated α-tocopherol decreased the hind paws edema, the leukocytes infiltration into femorotibial joints and the mRNA expression of pro-inflammatory cytokines in the tibial anterior muscle of arthritic rats, but the encapsulated compound was more effective. Free (i.p.) and encapsulated α-tocopherol decreased the high levels of reactive oxygen species in the brain and liver, but only the encapsulated compound decreased the levels of protein carbonyl groups in these organs. Both free (i.p.) and encapsulated α-tocopherol increased the α-tocopherol levels and the ratio of reduced to oxidized glutathione in these organs.
Conclusion: Both intraperitoneally administered free α-tocopherol and orally administered encapsulated α-tocopherol effectively improved inflammation and systemic oxidative stress in middle-aged arthritic rats. However, the encapsulated form should be preferred because the oral administration route does not be linked to the evident discomfort that is caused in general by injectable medicaments. Consequently, α-tocopherol-loaded polycaprolactone nanoparticles may be a promising adjuvant to the most current approaches aiming at rheumatoid arthritis therapy.

1. Introduction

Rheumatoid arthritis is a chronic and autoimmune inflammatory disease that affects primarily the joints and is associated with progressive disability and premature death.[1] This disease affects nearly 1.0% of the adult population worldwide.[2] The pathophysiology of arthritis involves an intense hyperplasia of synovial membrane with participation of proinflammatory cytokines and overproduction of reactive species.[3] Rheumatoid arthritis is systemic and, in addition to the joints, inflammation and oxidative stress affect other organs, such as lungs, liver and brain.[4–6] Metabolic changes are also prominent, such as muscle wasting, a condition known as rheumatoid cachexia.[7]

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Abbreviations

Alpha-tocopherol	α-tocopherol
ROS	reactive oxygen species
GSH	reduced glutathione
GSSG	oxidized glutathione
SOD	superoxide dismutase
FRAP	ferric reducing ability of plasma
AST	aspartate aminotransferase
ALT	alanine aminotransferase
NF-kB	nuclear factor kappa B
Nfr2	nuclear factor erythroid 2-related 2
TNFα	tumoral necrosis factor alpha
IL-1β	interleukin 1 beta; IL-6, interleukin 6

Adjuvant-induced polyarthritis is a T cell-mediated chronic inflammatory immunopathology in rats that shares many features with rheumatoid arthritis: synovial hyperplasia, systemic inflammation and cachexia.[8] In these animals, oxidative stress is also increased in many organs, such as brain and liver.[9–11] Metabolic modifications are also very common and in addition to cachexia significant alterations occur in the liver metabolism, such as reduced gluconeogenesis and increased glycolysis and fatty acids oxidation.[12–14] Particularly in the liver, where metabolic changes are pronounced, oxidative stress is more accentuated when compared to other organs.[10–12] In arthritic brains, the increased oxidative stress is also associated with decreased GSH/GSSG ratio and lower activity of antioxidant enzymes[10].

Alpha-tocopherol (α-tocopherol) is one of the major cellular antioxidants and belongs to the class of *sacrificial antioxidants*, which are consumed during the antioxidant process and need to be replaced because they cannot be efficiently recycled.[15] In the case of rheumatoid arthritis, the levels of α-tocopherol are decreased in blood and synovial fluid of patients, a condition that manifests even before the onset of the disease.[15,16] Supplementation with α-tocopherol, on the other hand, decreases oxidative stress in the blood of patients.[17] Similarly, mice with type II collagen-induced arthritis fed with a α-tocopherol-deficient diet present higher expression of synovial proinflammatory cytokines, which is normalized by subsequent supplementation with α-tocopherol.[18]

Considering the above, it would be expected that α-tocopherol would improve the articular inflammation and systemic oxidative stress of arthritic rats. In fact, a previous study showed that subcutaneously administered α-tocopherol decreases leukocyte infiltrations and the levels of proinflammatory cytokines in the injected paw of rats with adjuvant-induced mild arthritis (monoarthritis).[19] However, these effects only occurred at the dose of 600 mg kg^{-1}. If subcutaneously injected α-tocopherol is to be used as a complement in the therapeutic approach to rheumatoid arthritis and other chronic diseases, one should be aware that this implies in repeated injections for a relatively long period. Oral intake would be no doubt a much better solution for improving patient compliance. The lipophilic character of α-tocopherol allows it to easily permeate cellular membranes and to be rapidly absorbed, but at the same time, makes it difficult to interact with the gastrointestinal aqueous environment. For this reason, many strategies have been used to improve the aqueous solubility of α-tocopherol, including its introduction into nanoparticles prepared with biodegradable polyesters, such as poly-ε-caprolactone, what further allows the controlled release of the compound in the gastrointestinal tract.[20–22] This should improve the bioavailability of orally administered α-tocopherol and therefore decrease its effective dose.

The incidence of rheumatoid arthritis increases with age and it affects predominantly middle-aged people and elderly.[23] Likewise, the aging process itself is already associated with increased systemic oxidative stress and diminished immune cell functions (immunosenescence), which contribute to the development of rheumatoid arthritis.[24–27] Therefore, the present study investigated the effects of orally administered α-tocopherol-loaded polycaprolactone nanoparticles on articular inflammation and oxidative stress in the plasma, liver and brain of middle-aged rats with adjuvant-induced arthritis. These effects were compared with those of orally and intraperitoneally administered unencapsulated α-tocopherol.

2. Material and methods

2.1. Nanoparticles preparation

Nanoparticles of poly-ε-caprolactone (PCL) containing α-tocopherol were prepared by nanoprecipitation as previously described.[21] Briefly, in an ultrasonic bath, 0.14 g of PCL was dissolved in 4.0 mL of acetone at 50 °C. Then, 10.0 mL of lecithin solution in acetone:ethanol (60:40) was added to the initial solution and mixed with 0.80 g of α-tocopherol. This organic solution was then transferred, under moderate stirring, to an aqueous solution containing 50.0 mL of hydrophilic surfactant (Pluronic F68 15 g%). The milky suspension resulting from nanoencapsulation was left free of the organic solvent by evaporation in a water bath (45 °C) under stirring overnight. The α-tocopherol-loaded nanoparticles were finally obtained by lyophilization. Empty nanoparticles were prepared in the same way without addition of α-tocopherol. The nanoparticles size was homogeneous,[21] the encapsulation efficiency of α-tocopherol in the nanoparticles is around of 90%[21] and α-tocopherol loading was around of 10% (w/w).

2.2. Animals and induction of arthritis

Male *Holtzman* rats were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and kept in the Animal Care Unit of our laboratory under standard conditions. The animals were fed ad libitum with laboratory diet (Nuvilab®, Colombo, Brazil). For arthritis induction, 12 months aged animals (adults) were subcutaneously injected in the base of the left hind paw with 0.1 mL (500 μg) of Freund's adjuvant (heat inactivated *Mycobacterium tuberculosis*, derived from the human strain H37Rv), suspended in mineral oil.[31] Rats of similar ages were injected with mineral oil and served as controls. The procedures followed the guidelines of the Brazilian Council for the Control of Animal Experimentation (CONCEA) and were previously approved by the Ethics Committee for Animal Experimentation of the State University of Maringá (Protocol number CEUA 2495130916).

2.3. Experimental design

Two protocols of treatment were followed: (1) intraperitoneal administration of 100 mg kg^{-1} of unencapsulated α-tocopherol (free α-tocopherol), and (2) oral administration of free and encapsulated α-tocopherol (100 mg kg^{-1}). For protocol (1), rats were distributed into four groups: controls, to which corn oil (C-co/ip) or free α-tocopherol (C-α/ip) were intraperitoneally administered; and arthritic rats, to which corn oil (A-co/ip) or free α-tocopherol (A-α/ip) were intraperitoneally administered. Regarding to protocol (2), rats were distributed into six groups: control and arthritic rats to which nanoparticles (C-n and A-n), free α-tocopherol (C-α and A-α), or encapsulated α-tocopherol (C-nα and A-nα) were orally administered. Animals were daily treated five days before and 18 days

after arthritis induction and used on day 19. Free and encapsulated α-tocopherol were dissolved, respectively, in corn oil and water for administration. The curative treatment during 18 days after arthritis induction has been used because the chronic inflammatory manifestations are particularly evident between the 14th and 21st days after the induction[28–30]. A preventive treatment protocol has been also used because rheumatoid arthritis is intermittent and adjuvant-induced arthritis is a model of severe arthritis.[28]

2.4. Evaluation of the inflammatory response

Following adjuvant inoculation, the volume of both hind paws up to the tibiotarsal joint was measured by plethysmography over a 18-days period. The results were expressed in terms of increased paw volume in relation to the initial volume (volume at day 0). The appearance and severity of secondary lesions were also assessed from the 10th to the 18th day according to the score previously described.[32] Blood was collected by means of tail incision to count the number of circulating leukocytes. The total leukocytes recruited into the femorotibial joint cavity were additionally counted at the 19th day.

2.5. Tissue preparation

Fasted (12 h) rats were anesthetized with an overdose of sodium thiopental (100 mg kg^{-1} i.p.) and the peritoneal cavity was surgically exposed. Blood was then collected from cava vein and deposited into heparinized tubes. The liver and brain were subsequently removed and immediately freeze-clamped and stored in liquid nitrogen. Thereafter, the hind femorotibial joints were surgically exposed, articular cavities were washed with 40 μL of phosphate-buffered saline (PBS) solution containing 1 mM EDTA and exudates used for leukocyte count.

Blood was centrifuged at 9,000*g*/10 min, the supernatant was separated and stored in the dark at −80 °C. Freeze-clamped portions of the tissues were separately homogenized in a van Potter-Elvehjem homogenizer with 10 vol of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and an aliquot was separated for use as total homogenate. The remaining homogenate was centrifuged at 11,000*g* during 15 min and the supernatant separated.

2.6. Histopathological study

Additional groups of animals equally treated as described above were euthanized on day 19 with an overdose of anesthetic (100 mg/kg of sodium thiopental) and the left femorotibial joint of each rat was collected and fixed in 10% formalin for 48 h, decalcified in 10% EDTA (pH 7.4) for 21 days. The samples were afterwards processed for paraffin embedding. Semi-serial sections of 6 μm were made and stained with hematoxylin and eosin. For microscopic analysis, the sections were observed in a Nikon Eclipse® microscope (Tokyo, Japan) coupled with a Nikon camera (Ds-Fi1c, Shimjuku, Japan).

2.7. Measurement of cytokines mRNA expression

Animals were euthanized on day 19 after arthritis induction with an overdose of anesthetic (100 mg/kg of sodium thiopental i.p.) and the left tibial anterior muscle samples were collected and stored in liquid nitrogen at −80 °C pending total RNA extraction.[33] RNA was isolated from 100 mg frozen tissue using the U-Trizol reagent (Uniscience, BR). The RNA concentration was measured using a spectrophotometer at the wavelength of 260 nm (NanoDrop ND 1000 NanoDrop Technologies, Wilmington, DE). cDNA was synthetized using the QuantiNova® Reverse Transcription Kit (Qiagen, DE) and quantitation of the tissue expression of selected genes was done by quantitative PCR in a Lightcycler 96 (Roche) with "HOT FirePol® EvaGreen® qPCR Supermix" (Solis BioDyne). The GADPH gene was utilized as a reference gene. The $2^{-\Delta CT}$ method was used for the relative quantification analysis and data were expressed in arbitrary units (AU).[34]

2.8. Oxidative stress assays

The levels of protein carbonyl groups were measured by spectrophotometry using 2,4-dinitrophenylhydrazine as previously described.[35]

The levels of reactive oxygen species (ROS) were quantified in the supernatant of liver and brain homogenates by spectrofluorimetry with 2′,7′-dichloro-fluorescein diacetate (DCFH-DA).[36]

Reduced (GSH) and oxidized glutathione (GSSG) were measured spectrofluorimetrically (excitation at 350 nm and emission at 420 nm) by means of the *o*-phthalaldehyde (OPT) assay as previously described.[37]

The catalase activity was estimated by measuring the change in absorbance at 240 nm using H_2O_2 as substrate.[38] The activity of SOD was estimated by its capacity to inhibit pyrogallol autoxidation in alkaline medium at 420 nm.[39] One SOD unit was considered the quantity of enzyme that was able to promote 50% inhibition and results were expressed as units/mg protein.

The ferric reducing ability of plasma (FRAP) was measured by spectrophotometry (595 nm) using tripyridyltriazine (TPTZ) and ferric chloride ($FeCl_3$).[40]

Thiol contents were measured by spectrophotometry (412 nm) using DTNB (5,5′-dithiobis 2-nitrobenzoic acid) as previously described.[41]

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in the plasma using commercial kits (Gold Analisa®).

2.9. Levels of α-tocopherol in the liver and brain

The contents of α-tocopherol in liver and brain were determined by high-performance liquid chromatography (HPLC) analysis (Shimadzu, Japan). A freeze-clamped portion of hepatic and brain tissues were separately homogenized in a van Potter-Elvehjem homogenizer with 10 vol of ice-cold absolute ethanol and centrifuged at 9,500*g* during 10 min. An aliquot (0.5 mL) of supernatant was added in a falcon tube containing 1.5 mL of hexane, 1.5 mL of absolute ethanol, 1.0 mL of ultrapure water, 0.1 mL of sodium dodecyl sulfate (SDS 0.1 M) and 50 μL of butylated hydroxytoluene (BHT 50 mM). The mixture was vortexed for 1 min, kept for 1 h in the dark, vortexed again and centrifuged at 2,700*g* for 5 min. An aliquot of 1.0 mL of the hexanic fraction was dried under N_2 stream and resuspended in 500 μL of methanol for HPLC analysis. A reversed-phase C18 CLC-ODS column (5 μm, 150 × 4.6 mm i.d.; Shimadzu) protected with a CLC-ODS precolumn (5 μm, 4 × 3 mm i.d.; Phenomenex), was used with an isocratic mobile reverse phase (methanol:water, 98:2). The samples were eluted from the column with a flow rate of 1.5 mL/min. Detection was done spectrophotometrically at 260 nm.

2.10. Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of the GraphPad Prism Software (version 5.0). The statistical significance of the data was analyzed by means of ANOVA ONE-WAY and the Newman-Keuls post-hoc test was applied with the 5% level of significance ($p < 0.05$).

3. Results

3.1. Effects of alpha-tocopherol on inflammation

The intraperitoneal route of administration was chosen as reference because of the large surface area of the abdominal cavity and the abundant blood supply, features that allow rapid delivery to the whole body.[42] Fig. 1A and B show, respectively, the time-courses of the volume changes and the final volume increments (edema) of the injected paws. The initial volume of the hind paws before adjuvant injection was 1.85 ± 0.20 mL. Inflammatory reactions in the injected paws were observed at the first day and they were equal in all arthritic groups. From the second day the paw volumes of the α-tocopherol-treated arthritic groups tended to be smaller and at the 18th day (around 40% when compared to the corn oil or nanoparticles-treated arthritic rats; Fig. 1B).

The time-course of the volume changes and the final volume increments of the non-injected hind paws (contralateral paws) are shown, respectively, in Fig. 1C and D. The volume of the contralateral paw of all groups was not modified until day 10, when the paw volume of corn oil- and nanoparticles-treated arthritic animals progressively increased until day 18. The intraperitoneal administration of free α-tocopherol and oral administration of encapsulated α-tocopherol caused smaller increments (by 53%) in the contralateral paw volumes (Fig. 1D). The oral administration of free α-tocopherol did not decrease the edema of the contralateral paw.

The body weight before starting treatment was 540 ± 30 g and treatment of control rats did not change this parameter (Fig. 2A). Following the adjuvant injection, the body weight of all arthritic groups decreased progressively until day 19, when they were around 16% lower than that of control animals. Secondary lesions appeared at day 10 and reached the highest scores on day 18 for corn oil and nanoparticles-treated arthritic rats (Fig. 2B). These scores were lower only in arthritic rats intraperitoneally treated with free α-tocopherol.

The number of leukocytes recruited into the left femorotibial join cavity (joint of the injected paw) was around 85% higher than that of the leukocytes recruited into the contralateral joint cavity (Fig. 2C and D). The number of leukocytes in the contralateral joint cavity was decreased by approximately 25% in all α-tocopherol-treated arthritic groups. The number of leukocytes in the left femorotibial join cavity was decreased by approximately 15% in arthritic rats intraperitoneally and orally treated with free α-tocopherol, but the number was even smaller (−28%) in rats orally treated with encapsulated α-tocopherol. The number of circulating leukocytes on day 18 was increased four-fold in corn oil or nanoparticles-treated arthritic rats (compared to day zero) and treatment of all arthritic groups with α-tocopherol did not produce any decrease (Fig. 2E).

Considering that the number of leukocytes in the left femorotibial joint cavity was smaller in rats treated with encapsulated α-tocopherol (compared to rats intraperitoneally and orally treated with free α-tocopherol), histological analyses of inflammation and cartilage damage were performed in this joint. Fig. 3 shows the typical photomicrographs of knee joint sections that were stained with hematoxylin/eosin. In the control group (A) observe, in sagittal section, the joint of a control animal, with the articular surfaces of the femur (f) and tibia (t) delimited by the dotted line, the cruciate ligament (li), the synovial membrane (sm), with adipocytes in the subintima and the fibrous membrane of the joint capsule (ca) on the anterior surface of the joint. The articular

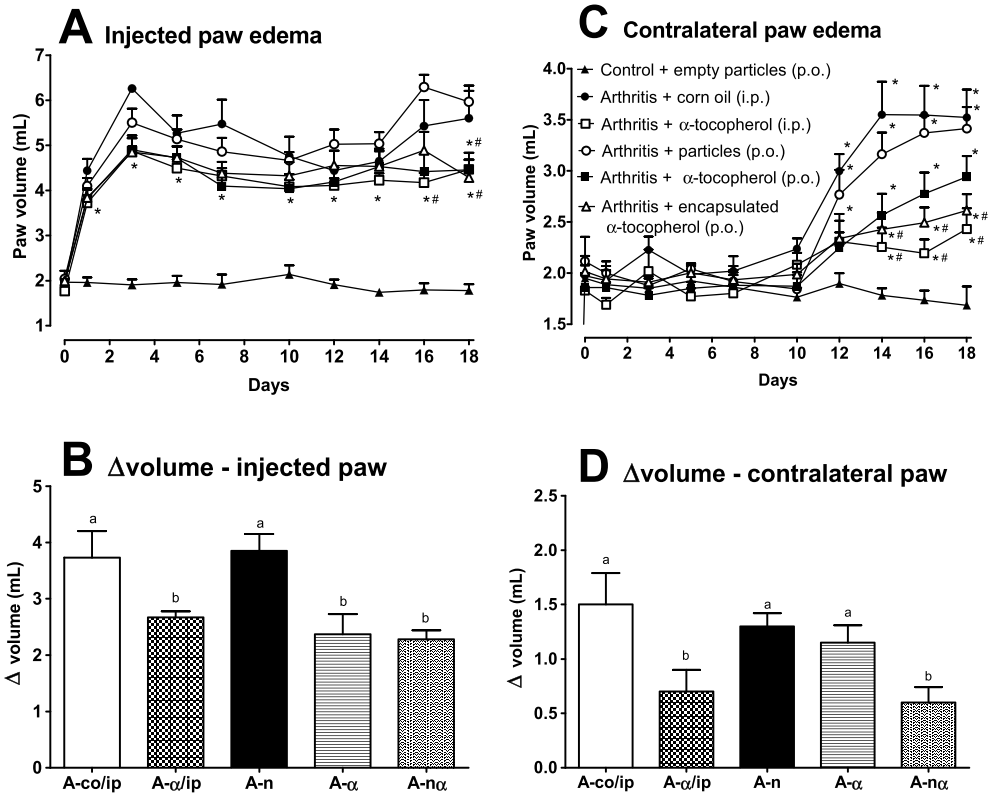

Fig. 1. *Effects of α-tocopherol on evolution of paw volume of arthritic rats.* Δ volume of paws (paw edema) = volume at day 18 - initial volume (day zero). A-co/ip, corn oil-treated rats (i.p.); A-α/ip, α-tocopherol-treated rats (i.p.); A-n, empty particles-treated rats (p.o.); A-α, unencapsulated α-tocopherol-treated rats (p.o.); A-nα, encapsulated α-tocopherol-treated rats (p.o.). The values are the mean ± SEM of 5–7 animals. PANEL A and C: *$p < 0.05$: for difference from controls + empty particles; #$p < 0.05$: for difference from arthritis + empty particles. PANEL B and D: Values with different superscript letters are different.

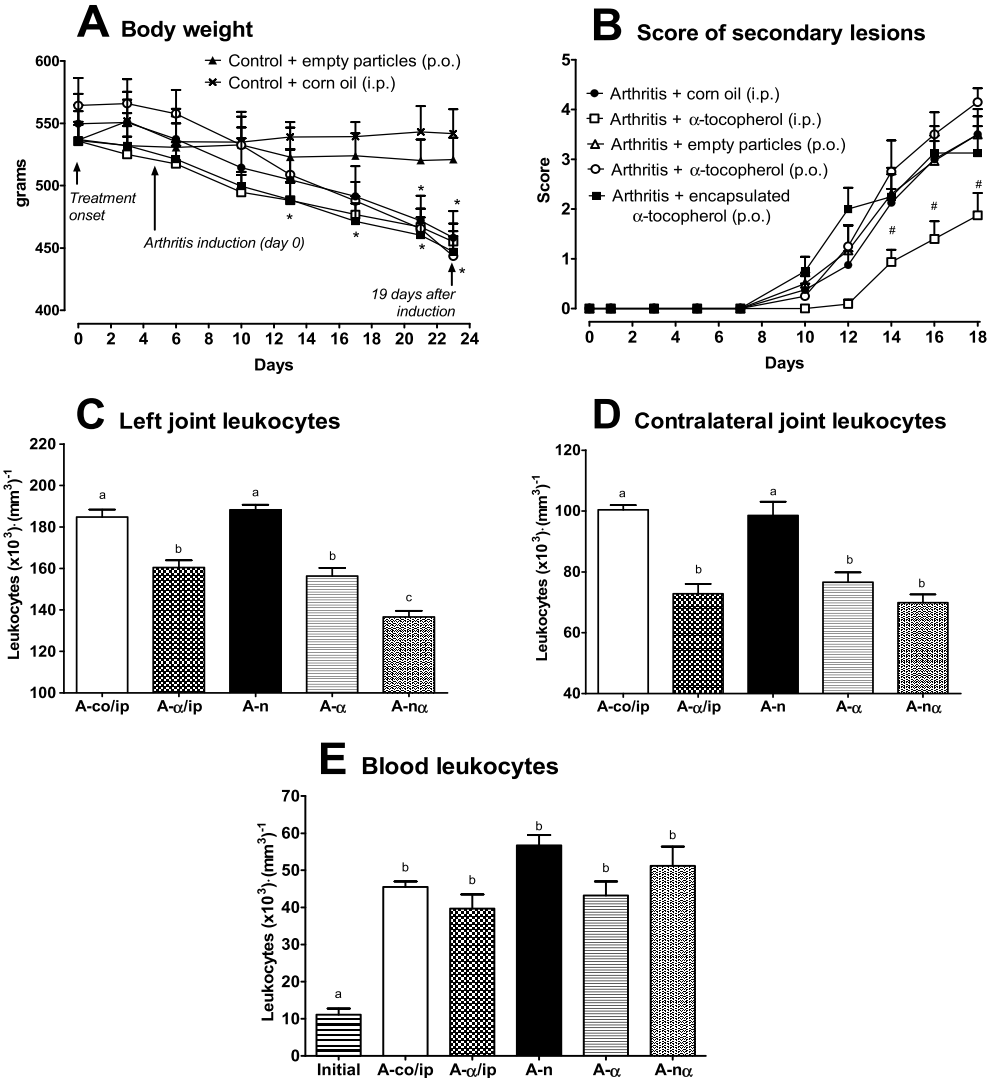

Fig. 2. *Effects of α-tocopherol on body weight, arthritis score and number of blood and articular leukocytes of arthritic rats.* Treatment of the animals was initiated 5 days before arthritis induction and maintained for 18 days after (PANEL A). The number of articular leukocytes was measured in the femorotibial hind joints at day 19. A-co/ip, corn oil-treated rats (i.p.); A-α/ip, α-tocopherol-treated rats (i.p.); A-n, empty particles-treated rats (p.o.); A-α, unencapsulated α-tocopherol-treated rats (p.o.); A-nα, encapsulated α-tocopherol-treated rats (p.o.). The values are the mean ± SEM of 5–7 animals. PANEL A and B: *p < 0.05: for difference from controls; #p < 0.05: for difference from arthritis + empty particles. PANEL C, D and E: Values with different superscript letters are different.

cartilage had smooth surfaces with intact layers, without inflammation or bone loss (Fig. 3A). In untreated arthritic animals (B) the articular surface is inflamed and swollen, and fibrous connective tissue (fi) replaces the articular surfaces. In (C), the synovial membrane detail shows the loss of the intimal monolayer (arrows) and the replacement of subintimal adipocytes by granulation tissue, characteristic of the *pannus* (Fig. 3B and C). In the group orally treated with encapsulated α-tocopherol, the joint did not show the typical changes of arthritis, except for the synovial membrane which presented a fibrotic band adjacent to the intima (in the inset of Fig. 3D). The other morphological characteristics were similar to those of the control group (Fig. 3D). The femorotibial joints of rats treated orally with free α-tocopherol showed inflammation and fibrosis in the joint space and in the subintima of the synovial membrane. The collagen fibers deposited in the subintima were thicker and organized in parallel bundles (Fig. 3E and F). After intraperitoneal treatment with free α-tocopherol, no inflammation or morphological changes in joint tissues were observed (Fig. 3G and H). Histological analyses showed that rats that received encapsulated α-tocopherol orally or free α-tocopherol intraperitoneally presented morphological characteristics similar to those of control animals.

Considering that the tibial muscle is directly associated with the knee joints and the cytokines levels are reported to increase in this tissue of rats with osteoarthritis,[33] the mRNA expressions of the proinflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumoral necrosis factor alpha (TNFα) were assessed in the anterior region of this muscle. The results are shown in Fig. 4. The levels of IL-1β, IL-6 and TNFα mRNA were respectively 13, 20 and 40 times higher in the tibial anterior muscle of nanoparticles-treated arthritic rats (compared to the controls). The levels of IL-1β and IL-6 mRNA were approximately 50% and 85% lower in rats intraperitoneally and orally treated with free α-tocopherol, respectively (when compared to non-treated arthritic rats). Treatment with encapsulated α-tocopherol, however, maintained the control levels (Fig. 4A and B). The levels of TNFα mRNA were approximately 75% and 85% lower, respectively, in rats intraperitoneally and orally treated with free α-tocopherol (compared to non-treated arthritic rats). Treatment with encapsulated α-tocopherol, however, maintained the control levels (Fig. 4C).

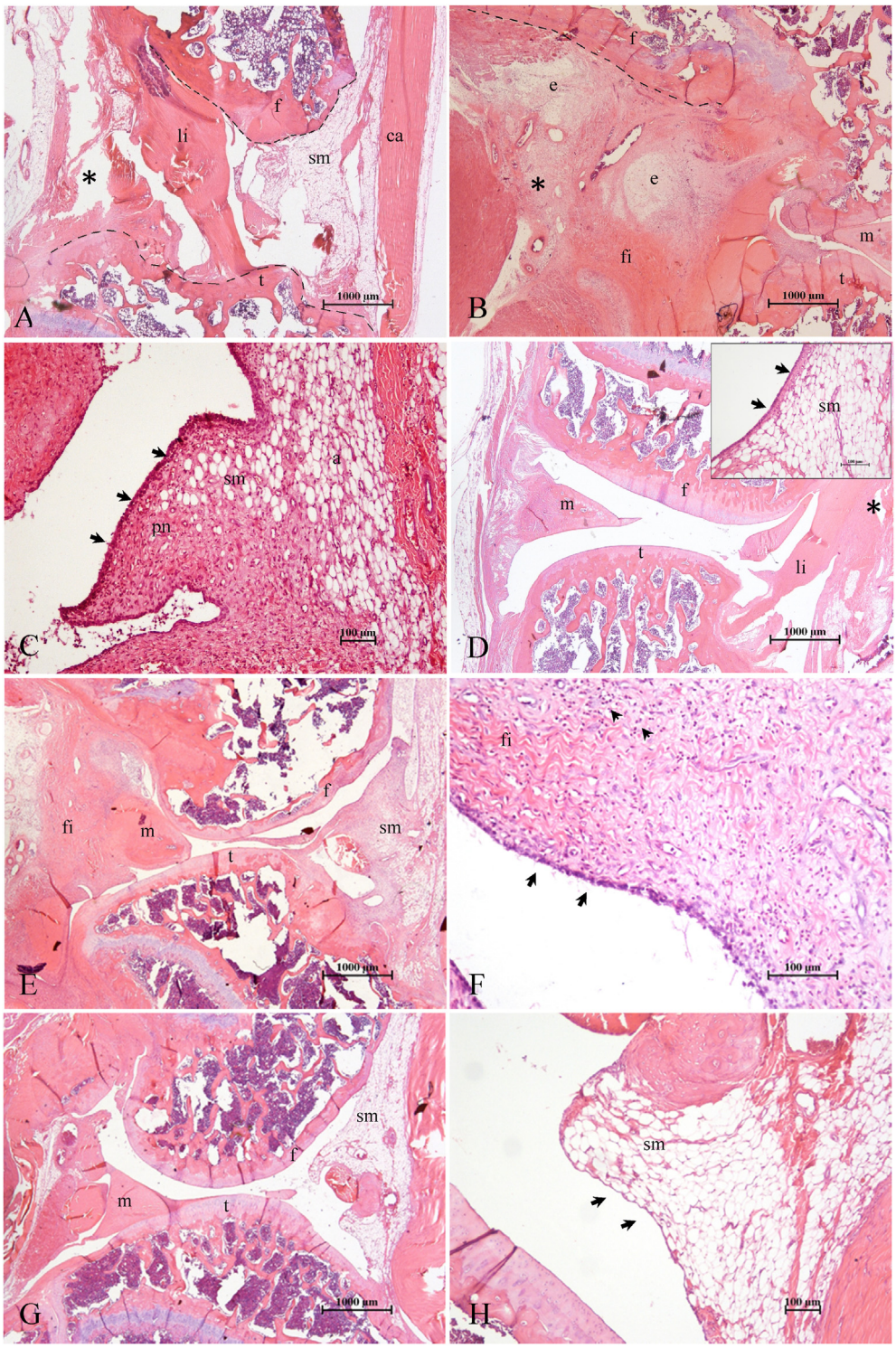

Fig. 3. Photomicrograph of the femorotibial joint of rats. (A) Control, (B and C) arthritic, (D) arthritic treated with encapsulated α-tocopherol, orally, (E and F) arthritic treated with free α-tocopherol, orally, and (G and H) arthritic treated with free α-tocopherol, intraperitoneally. The inset in (D) details the band of fibrotic tissue adjacent to the intima in the synovial membrane. Arrows indicate intimal layer and arrowheads indicate the presence of inflammatory cells. (*) indicate posterior face or articular surface. Legend: (f) femur; (t) tibia; (li) cruciate ligament; (sm) synovial membrane; (ca) joint capsule; (e) edema; (fi) fibrosis; (m) meniscus; (pn) *pannus*; (a) adipocyte; (pn) *pannus*. Staining: hematoxylin and eosin.

3.2. Effects of α-tocopherol on systemic oxidative stress

Fig. 5 illustrates the oxidative status of the plasma. The levels of protein carbonyl groups were 30% higher in the nanoparticles-treated arthritic rats (compared to the controls; Fig. 5A). These levels were decreased by 15% in all α-tocopherol treated arthritic groups. The levels of thiol groups were quite lower in the plasma of nanoparticles-treated arthritic rats (Fig. 5B). Alpha-tocopherol did not increase the levels of thiol groups irrespective of the way by which it was administered. The ferric reducing ability of the plasma

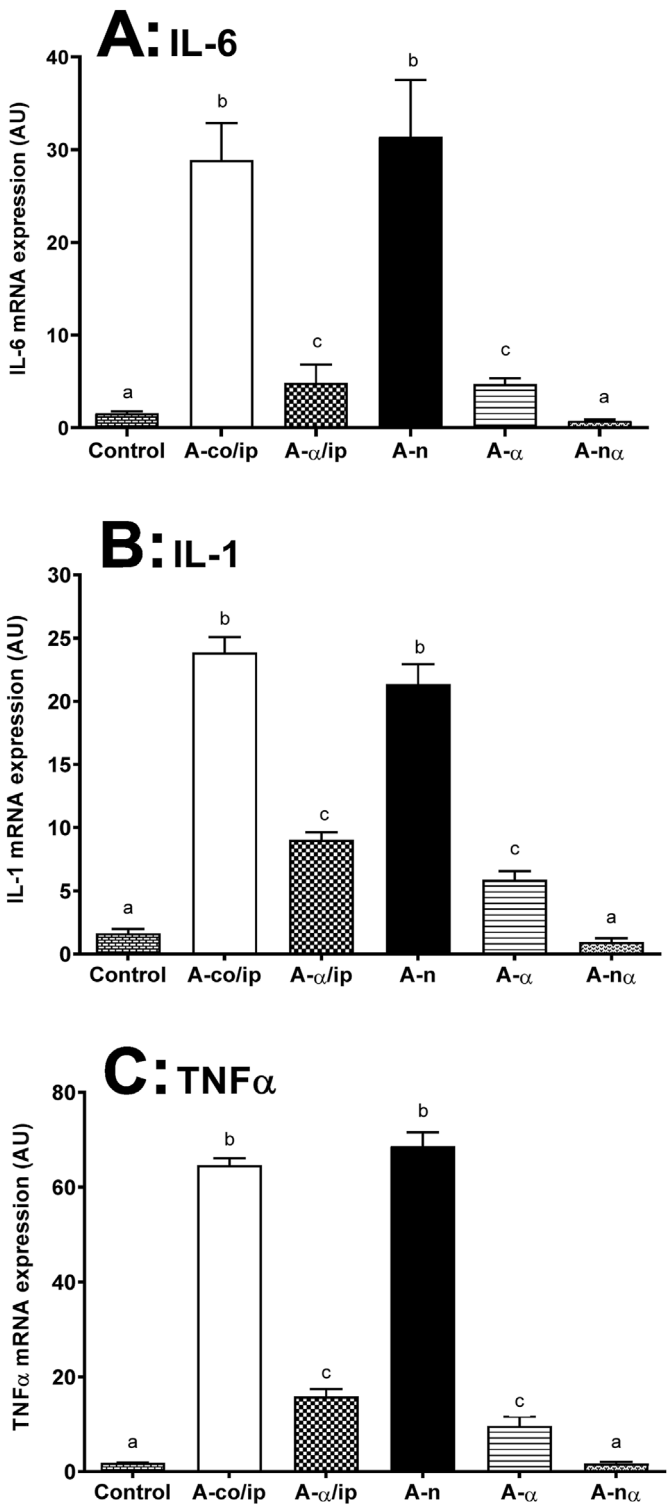

Fig. 4. *Effects of α-tocopherol on cytokines mRNA expression in the* tibial anterior *muscle.* Control, corn oil-treated control rats; A-co/ip, corn oil-treated arthritic rats (i.p.); A-α/ip, free α-tocopherol-treated arthritic rats (i.p.); A-n, empty particles-treated arthritic rats (p.o.); A-α, α-tocopherol-treated arthritic rats (p.o.); A-nα, encapsulated α-tocopherol-treated arthritic rats (p.o.). Values are the means ± SEM of six animals. Values with different superscript letters are statistically different.

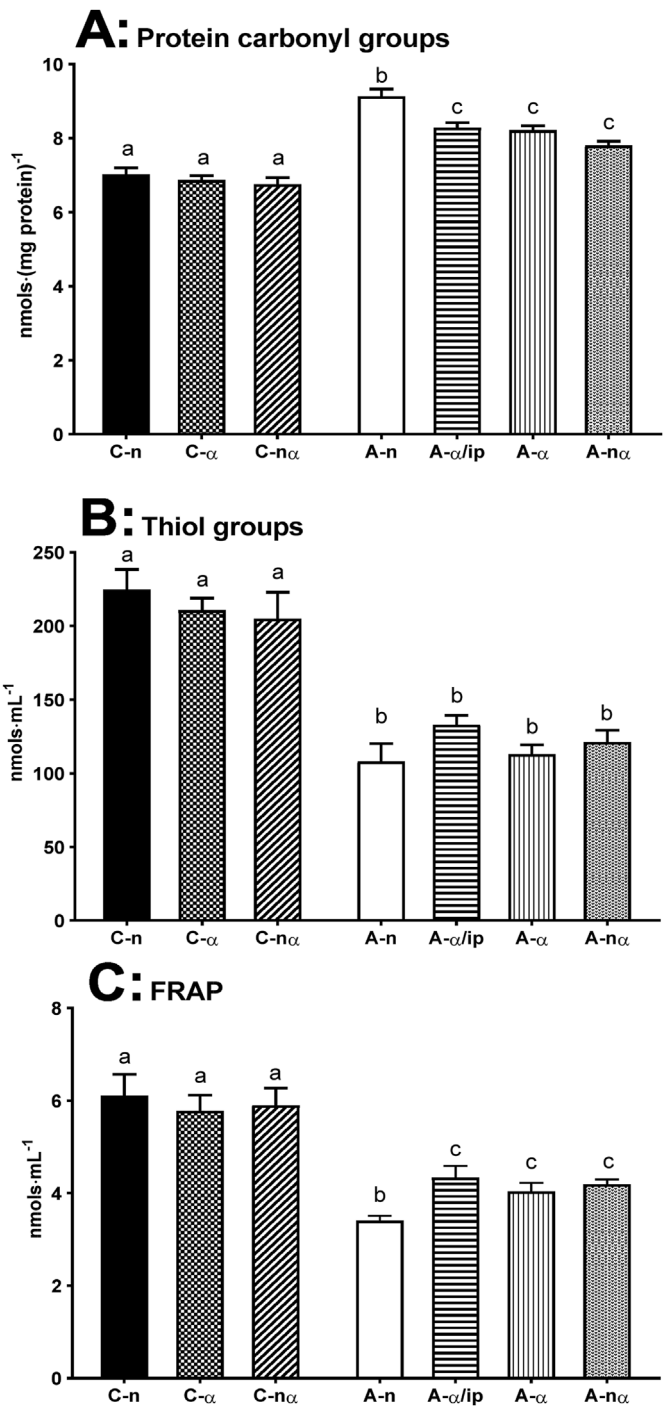

Fig. 5. *Effects of α-tocopherol on oxidative status in the plasma.* C-n, empty particles-treated control rats (p.o.); C-α, unencapsulated α-tocopherol-treated control rats; C-nα encapsulated α-tocopherol-treated control rats; A-n, empty particles-treated arthritic rats (p.o.); A-α/ip, unencapsulated α-tocopherol-treated arthritic rats (i.p.); A-α, unencapsulated α-tocopherol-treated arthritic rats (p.o.); A-nα, encapsulated α-tocopherol-treated arthritic rats (p.o.). The values for control and arthritic rats treated with corn oil i.p. were not different from C-n and An, respectively, and therefore were omitted. Values are the means ± SEM of five animals. Values with different superscript letters are statistically different.

(FRAP) of arthritic rats was only half of that one found in control animals (Fig. 5C). Intraperitoneally administered α-tocopherol and both orally administered free and encapsulated α-tocopherol increased FRAP of arthritic rats equally by 22%.

Fig. 6 illustrates the oxidative status of the liver. The levels of protein carbonyl groups and ROS were, respectively, 40 and 100% higher in nanoparticles-treated arthritic rats (compared to the controls; Fig. 6A and B). The hepatic levels of ROS were decreased

by 20–30% in all α-tocopherol-treated arthritic groups, but only the orally administered encapsulated α-tocopherol decreased the levels of protein carbonyl groups in arthritic livers (−15%). The activity of catalase was significantly lower in the nanoparticles-treated arthritic rats and treatment of all groups with α-tocopherol had no effect on this enzyme activity (Fig. 6C). The activity of SOD was not different in control and arthritic groups. The hepatic levels of GSH were 30% lower in the nanoparticles-treated arthritic rats (compared to the controls) and treatment of all arthritic groups with α-tocopherol reestablished the GSH levels (Fig. 6E). Only intraperitoneal administration of α-tocopherol and oral administration of encapsulated α-tocopherol reestablished, in arthritic rats, the low ratio of GSH/GSSG (Fig. 6F).

Fig. 7 illustrates the oxidative status of the brain. The levels of protein carbonyl groups and ROS were, respectively, 25 and 50% higher in nanoparticles-treated arthritic rats (compared to the controls; Fig. 7A and B). Treatment of arthritic animals with α-tocopherol reestablished the control levels of ROS of all arthritic groups, but only orally administered encapsulated α-tocopherol decreased the levels of protein carbonyl groups in the arthritic condition (−14%). Catalase activity was 25–35% lower in nanoparticles-treated arthritic rats and only the oral administration of encapsulated α-tocopherol improved this enzyme activity (Fig. 7C). SOD activity was nearly 30% lower in nanoparticles-treated arthritic rats but only intraperitoneally administered α-tocopherol improved its activity. The GSH levels and the GSSH/GSSG ratio were, respectively, 30 and 40% lower in nanoparticles-treated arthritic rats. Intraperitoneal administration of α-tocopherol and oral administration of encapsulated α-tocopherol reestablished the control levels of GSH and the GSH/GSSG ratio (Fig. 7E and F).

3.3. Plasma AST/ALT and α-tocopherol content in the liver and brain

The activities of AST and ALT were measured in the plasma to evaluate a possible hepatic damage. The activities of both enzymes were not modified in the plasma of all groups (Fig. 8A). Fig. 8B and C show the content of α-tocopherol in the brain and liver, respectively. In animals that did not receive α-tocopherol, its content in both liver and brain were similar and relatively low. When α-tocopherol was intraperitoneally administered its contents were 11- and 15-fold higher in the liver of control and arthritic rats,

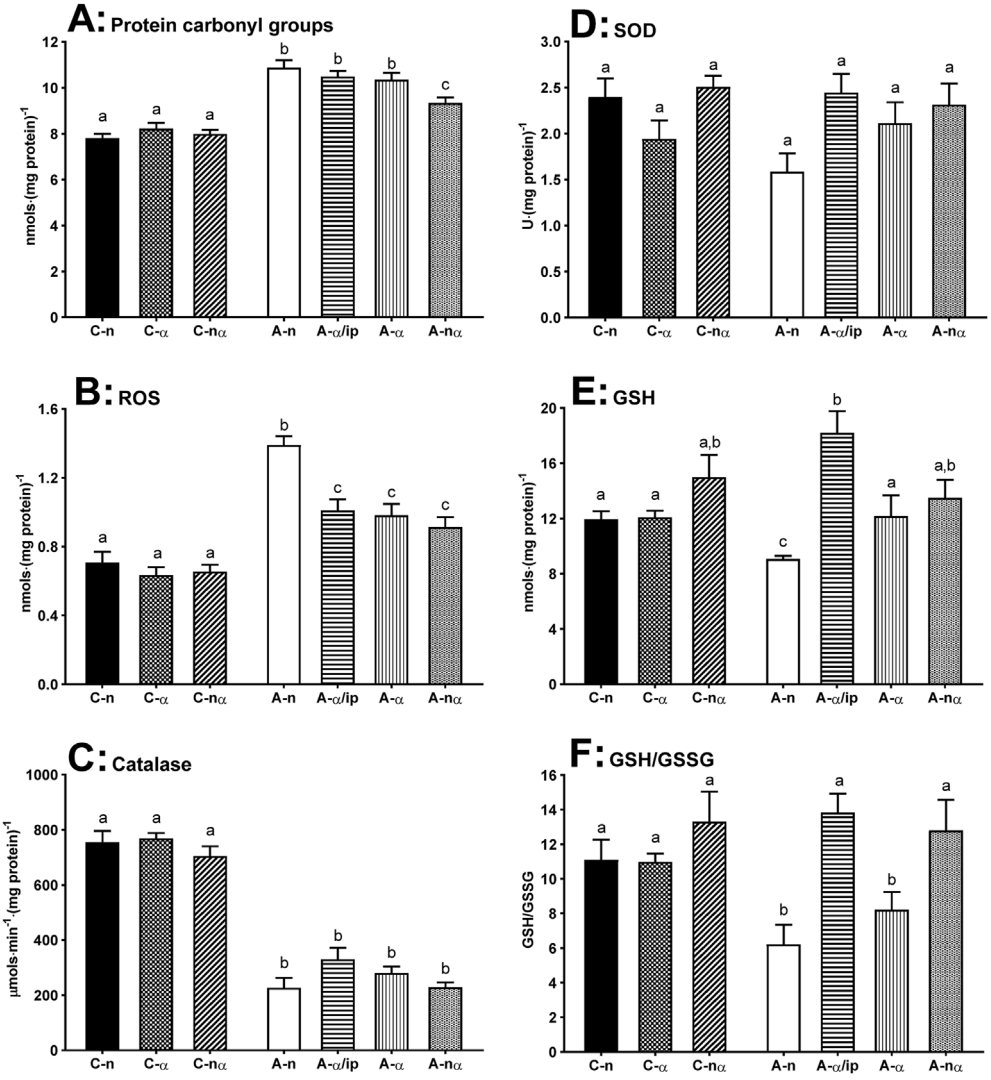

Fig. 6. *Effects of α-tocopherol on oxidative status of the liver.* C-n, empty particles-treated control rats (p.o.); C-α, unencapsulated α-tocopherol-treated control rats; C-nα encapsulated α-tocopherol-treated control rats (p.o.); A-n, empty particles-treated arthritic rats (p.o.); A-α/ip, unencapsulated α-tocopherol-treated arthritic rats (i.p.); A-α, unencapsulated α-tocopherol-treated arthritic rats (p.o.); A-nα, encapsulated α-tocopherol-treated arthritic rats (p.o.). The values for control and arthritic rats treated with corn oil i.p. were not different from C-n and An, respectively, and therefore were omitted. Values are the means ± SEM of five animals. Values with different superscript letters are statistically different.

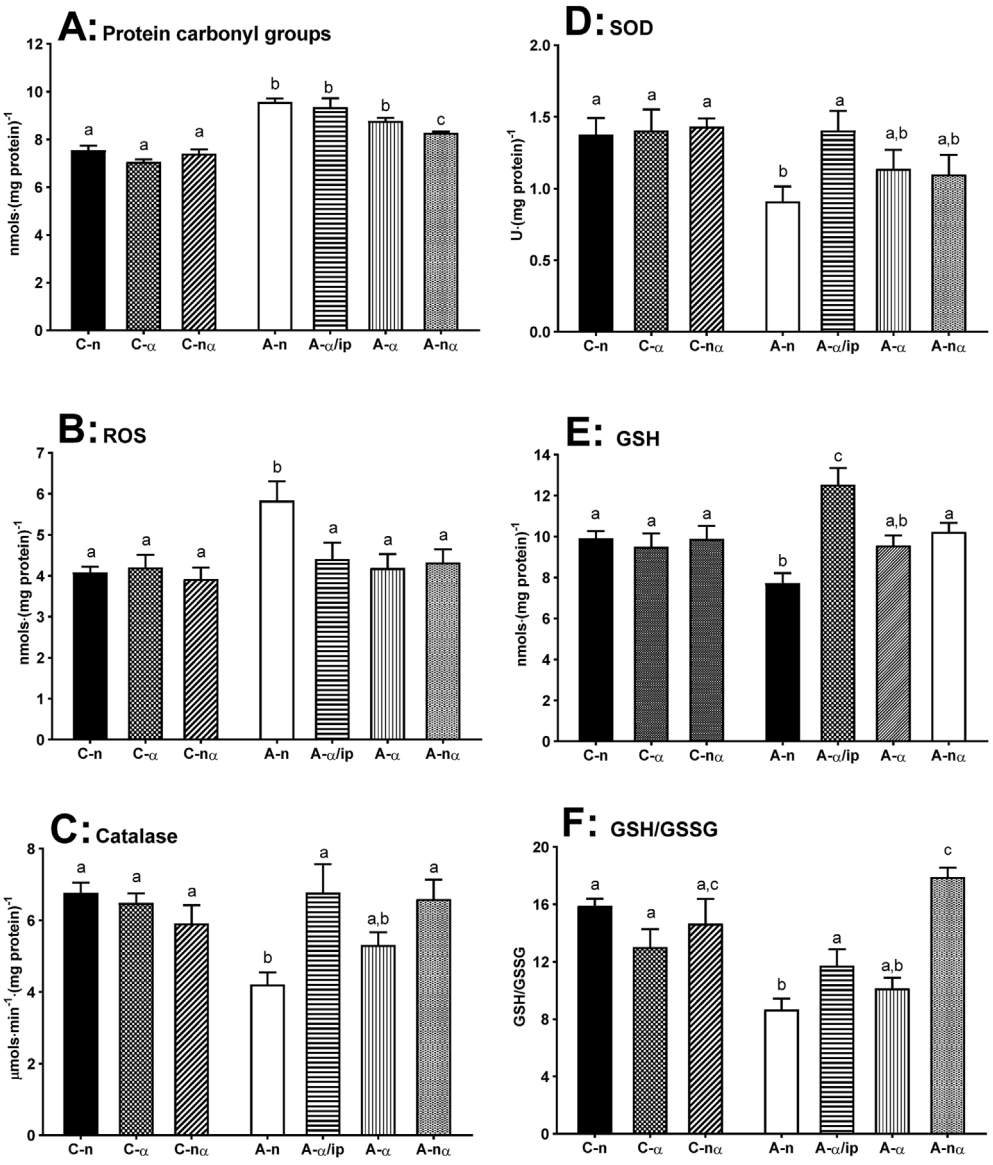

Fig. 7. *Effects of α-tocopherol on oxidative status of the brain.* C-n, empty particles-treated control rats (p.o.); C-α, unencapsulated α-tocopherol-treated control rats; C-nα encapsulated α-tocopherol-treated control rats (p.o.); A-n, empty particles-treated arthritic rats (p.o.); A-α/ip, unencapsulated α-tocopherol-treated arthritic rats (i.p.); A-α, unencapsulated α-tocopherol-treated arthritic rats (p.o.); A-nα, encapsulated α-tocopherol-treated arthritic rats (p.o.). The values for control and arthritic rats treated with corn oil i.p. were not different from C-n and An, respectively, and therefore were omitted. Values are the means ± SEM of five animals. Values with different superscript letters are statistically different.

respectively, but only 3-fold higher in the brain of both control and arthritic rats. When orally administered in the free form, the content of α-tocopherol was approximately 2-fold higher in the liver of control rats and approximately 2.5-fold in arthritic rats. When loaded nanoparticles were orally administered, on the other hand, the content of α-tocopherol was 3- and 6-fold higher in the liver of control and arthritic rats, respectively. In the brain, oral administration of the free form of α-tocopherol failed to produce a significant increase, but a 2-fold increase was found in arthritic rats. On the other hand, when loaded nanoparticles were orally administered the content of α-tocopherol in the brain was 3-fold higher in both control and arthritic rats.

4. Discussion

Adjuvant-induced arthritis is severe in rats and it shares several features of advanced rheumatoid arthritis.[8] The following basic characteristics of arthritic rats must be taken into account when interpreting the actions of both free and encapsulated α−tocopherol. The injection of the complete Freund's adjuvant in one of the hind paws causes a local inflammatory reaction and these animals also present widespread inflammatory manifestations, particularly between the 14th and 21st days after arthritis induction, when the immunological reaction develops in all paws (polyarthritis), joints and many organs.[29,30,43,44] The changes that are observed in this model are associated with intense cell migration and proinflammatory mediator release. Local joint cells (synoviocytes, macrophages, fibroblasts, chondrocytes) and recruited leukocytes produce and release inflammatory mediators, such as cytokines (TNFα, IL-1β, IL-6, among others), arachidonic acid metabolites, ROS and proteolytic enzymes.[30] All these enzymes and mediators participate in the development and progression of arthritis by stimulating leukocyte migration, joint and systemic inflammation, and articular tissue damage.

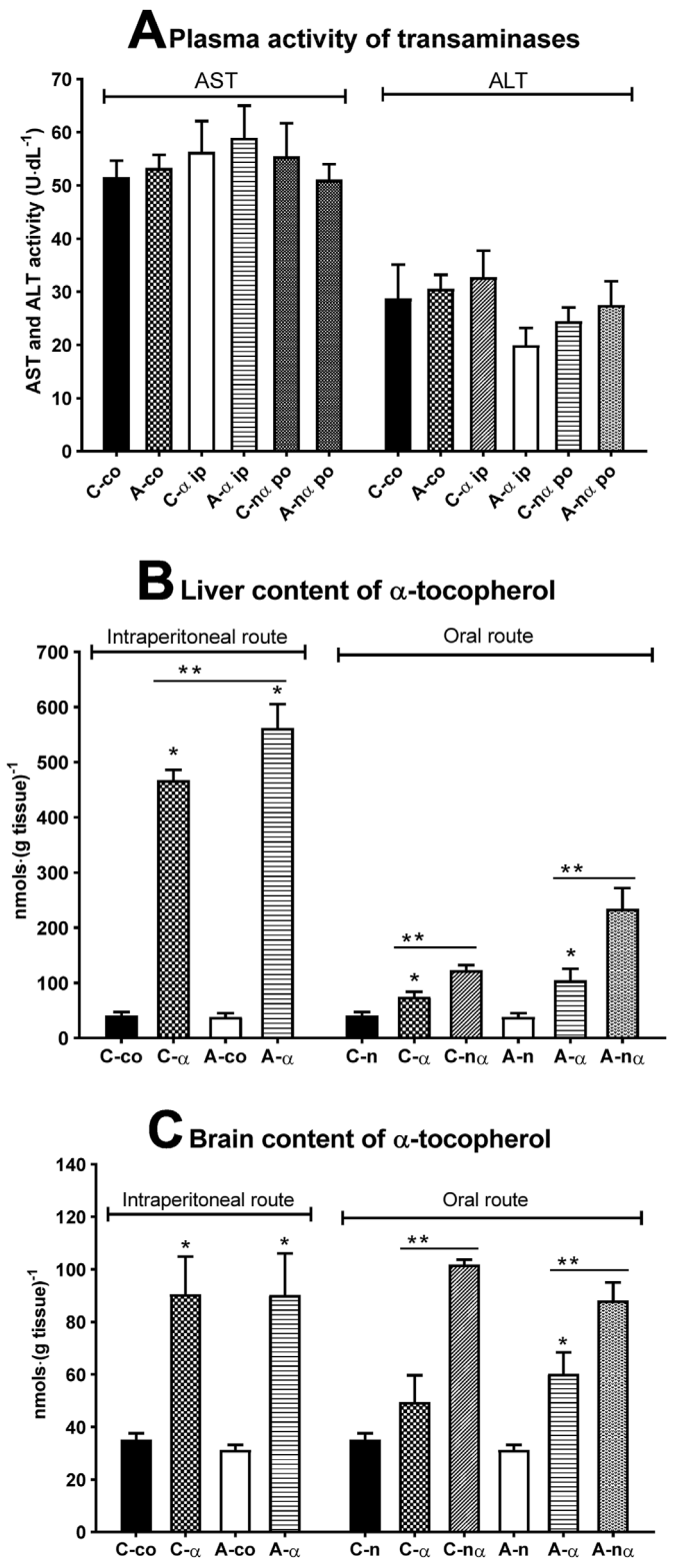

Fig. 8. *Activity of AST and ALT in the plasma (Panel A) and the content of α-tocopherol in the liver (Panel B) and brain (Panel C).* C-co, corn oil-treated control rats (i.p.); C-α, unencapsulated α-tocopherol-treated control rats (i.p. or p.o.); A-co, corn oil-treated arthritic rats (i.p.); A-α, unencapsulated α-tocopherol-treated arthritic rats (i.p. or p.o.); C-n, empty particles-treated control rats (p.o.); C-nα encapsulated α-tocopherol-treated arthritic rats; A-n, empty particles-treated arthritic rats (p.o.); A-nα, encapsulated α-tocopherol-treated arthritic rats (p.o.). Values are the means ± SEM of five animals. The symbol * stands for values differing from C-co or A-co or from C-n or A-n (oral route; $p \leq 0.05$); **indicates values differing as indicated by the horizontal bars.

With respect to the effect of α-tocopherol on arthritis, the actions of the intraperitoneally administered free compound, at the dose of 100 mg kg^{-1}, were considered to represent an adequate comparison basis. This conclusion was based on the fact that the intraperitoneal route results in high drug delivery rates and, therefore, in high cellular concentrations of α-tocopherol. Corroborating this, the intraperitoneally administered α-tocopherol was effective in decreasing the articular inflammation, joint damage, paw edema, the levels of proinflammatory cytokines in the tibial anterior muscle and oxidative stress (partly at least) in the liver, brain and plasma. Actually a previous study has already shown that subcutaneously administered α-tocopherol decreases the levels of IL-1β, TNFα and H_2O_2 in the injected paw of rats with mild adjuvant-induced arthritis (monoarthritis).[19] The latter is induced in rats by the administration of a low dose of the complete adjuvant, which is enough to cause only local arthritis in the injected paw.[28] This effect was observed only at a relatively high dose of subcutaneously injected α-tocopherol (600 mg kg^{-1}), which did not decrease the paw edema. In addition to polyarthritis, the animal model used in the present study also develops intense cachexia and presents a pronounced systemic oxidative stress that affects other organs, such as liver and brain.[11,45] The more pronounced effectiveness found with a lower dose of intraperitoneally injected α-tocopherol (100 mg kg^{-1}) is probably associated with the route of administration, which allows enhanced delivery and corporeal distribution of compounds when compared to the subcutaneous route.[42] In fact, when intraperitoneally administered, the content of α-tocopherol was substantially increased in both liver and brain. This increase was more pronounced in the liver when compared to the brain, however, a phenomenon probably related to the high vascularization of the peritoneal region that drains into the hepatic portal vein. The dose of 100 mg/kg would correspond to a human dose of approximately 15 mg/kg as given by the body surface area normalization method,[46] a value still within the recommended limit of α-tocopherol for adults.[47]

In spite of all the apparently beneficial effects of α-tocopherol injected by the intraperitoneal route that were described above, this route of administration would not be recommendable because of the obvious discomfort that it causes especially when practised for long periods of time. In this respect, our findings with orally administered α-tocopherol-loaded poly-ε-caprolactone nanoparticles present new perspectives in terms of the use of the compound as a possible adjuvant in the treatment of arthritis. It cannot be overseen that even orally administrated free α-tocopherol at the dose of 100 mg kg^{-1} as a corn oil solution was effective to improve several manifestations of arthritis. Effectiveness was in some cases even similar to that of the intraperitoneal injection. However, the orally administered free α-tocopherol was unable to decrease the contralateral paw edema and the levels of GSH in liver and brain. Furthermore, a pronounced inflammation and fibrosis in the joint space was still observable in arthritic rats treated orally with free α-tocopherol. All this is possibly related to the lower concentrations of α-tocopherol in the tissues when compared with those that were achieved when the compound was injected intraperitoneally. On the other hand, the α-tocopherol-loaded nanoparticles, which were also orally administered, presented a substantially increased effectiveness on inflammation and systemic oxidative stress. In addition, administration of encapsulated α-tocopherol to arthritic rats resulted in morphological characteristics similar to those of control animals, an action that was also observed when free α-tocopherol was administered via the intraperitoneal route. In some cases the effectiveness of orally administered encapsulated α-tocopherol proved even to be higher than that of the intraperitoneally injected free compound. For instance,

encapsulated α-tocopherol decreased even more the articular infiltration of leukocytes in the left femorotibial joint and the expression of proinflammatory cytokines in the tibial anterior muscle (Figs. 2C and 4A-C), whereas the injection of free α-tocopherol did not result in improved levels of protein carbonyl groups in the liver and brain (Figs. 6A and 7A). In addition, the orally administered α-tocopherol-loaded nanocapsules were the only ones that improved the catalase activity in the brain (Fig. 7C).

In relation to systemic oxidative stress it was evaluated in the liver and brain because it has been reported to be very pronounced in these organs of arthritic rats. This occurs in consequence of both a stimulated pro-oxidant system and a deficient antioxidant defense, with predominance of the latter as indicated by the strongly diminished activities of catalase and GSH levels.[10,11,45] Both phenomena seem to be caused by cytokines released into the synovium which may also reach the systemic circulation.[10,11] The levels of protein carbonyl groups were measured as markers of oxidative injury to proteins and FRAP and thiol groups were assayed as antioxidant markers in the plasma. Regarding the mechanism of antioxidant action, α-tocopherol may decrease the oxidative stress by acting directly as free radical scavenger (1), by stimulating the endogenous antioxidant system (2)[48] or by decreasing the inflammatory process (3). In the present study, the well-known ROS scavenger property of α-tocopherol (1) is certainly contributing to some extent for decreasing oxidative stress in the liver, brain and plasma. However, other antioxidant mechanisms must be evolved in the process, such as a stimulation of the endogenous antioxidant system (2). The latter hypothesis is substantiated by the observation that the tocopherols in general, including α-tocopherol, antagonize the inhibition of the nuclear factor erythroid 2-related 2 (Nfr2), which upregulates antioxidant defense genes, particularly those of enzymes related to the synthesis of GSH.[48] Furthermore, the anti-inflammatory activity of α-tocopherol (3) is certainly also contributing for decreasing oxidative stress. Supporting this conclusion is the observation that α-tocopherol decreased the levels of TNFα, IL-1β and IL-6 in the tibial anterior muscle of arthritic rats, which have been associated to the increase of leukocytes infiltration and ROS release in the joints and also, systemically, in the liver and brain.[10,11] At this regard, the nuclear factor NF-kB may be activated by oxidative stress and the effect of α-tocopherol has also been associated to the inhibition of the activation of NF-kB that, in turn, reduces oxidative stress and cytokines release.[49,50] It seems, thus, that α-tocopherol is not solely a potent antioxidant, but that it also has a pronounced anti-inflammatory activity, mainly by decreasing cytokine release in leukocytes.

Although α-tocopherol-loaded poly-ε-caprolactone nanoparticles have been already characterized and its antioxidant effect demonstrated *in vitro*,[20–22] the present study shows that this formulation is also effective *in vivo*. The higher effectiveness of α-tocopherol-loaded polycaprolactone nanoparticles is probably related not only to the improvement of the solubility of α-tocopherol in the aqueous medium, but also because encapsulation introduces factors that can improve the releasing profile of α-tocopherol in the gastrointestinal tract. These factors are the rates of disintegration and dissolution of the nanoparticles. In fact, a similar formulation containing α-tocopherol carried into polycaprolactone nanoparticles, when examined *in vitro*, exhibited a controlled release along 140 h with an initial burst during the first 20 h[20]. *In vivo,* this is likely to maintain a steady absorption of α-tocopherol throughout the treatment period. In this regard it is perhaps significant that the content of α-tocopherol was equally increased in the brain of rats treated orally with encapsulated α-tocopherol and intraperitoneally with free α-tocopherol.

Some results of the present study show that α-tocopherol given orally or injected intraperitoneally are apparently not effective at improving some inflammatory and oxidative parameters of arthritis. The body weight loss of arthritic animals, for example, was not prevented by α-tocopherol. It should be noted, however, that arthritic cachexia has been reported to be refractory to treatment and even classic anti-inflammatory drugs do not prevent these symptoms of adjuvant-induced polyarthritis in rats.[26,44,51] Similarly, the levels of thiol groups in the plasma of arthritic rats were not improved by both intraperitoneally and orally administered α-tocopherol. It should be noted, however, that thiol groups are decreased in the plasma of polyarthritic rats mainly in consequence of the considerably decreased levels of plasma albumin,[11,28] which alone accounts for approximately 70% of the plasma antioxidant capacity and 80% of all plasma thiol groups.[28,52] In this regard, even compounds that improve systemic inflammation and oxidative stress of arthritic rats do not improve the levels of thiol groups and albumin in the plasma of arthritic rats.[13,51] The FRAP assay offers a putative index of antioxidant or reducing potential of plasma[38] and, thus, the increase of FRAP in α-tocopherol-treated arthritic rats can occur as the result of a direct free radical scavenger action of α-tocopherol.

A final question to be approached refers to toxicity. Our results do not reveal appreciable hepatoxicity for poly-ε-caprolactone nanoparticles. This corroborates the report about absence of hepatotoxicity and nephrotoxicity for lipid-core nanoparticles containing a polymeric wall of poly(ϵ-caprolactone) after subchronic treatments (28 days).[53] Brain toxicity was also not found in spite of the fact that polycaprolactone polymers are reported to permeate the brain-blood barrier.[54]

5. Conclusion

The results of the present study show that articular inflammation and systemic oxidative stress in middle-aged arthritic rats can be improved by administering α-tocopherol. Oral administration of free α-tocopherol is less effective when compared to the intraperitoneal injection of free α-tocopherol. The oral administration of the encapsulated form of α-tocopherol, on the other hand, is clearly superior to the administration of the free form and slightly superior to the intraperitoneal injection of the same free compound. In the latter case, the encapsulated form of α-tocopherol should be clearly preferable because oral administration does not present the inconveniencies of the repeated injections of free α-tocopherol. The enhanced effectivity of the encapsulated form over the free form when given orally seems to be related to the higher amounts of α-tocopherol that are delivered to the tissues, as evidenced by the higher contents of the compound in the liver and brain of arthritic rats. The main anti-inflammatory mechanism of α-tocopherol seems to result from an inhibition of the production and release of proinflammatory cytokines. Inhibition of cytokines production and release also results in a diminution of oxidative stress, which can be further reduced by a direct free-radical scavenging activity of α-tocopherol. Consequently, α-tocopherol-loaded polycaprolactone nanoparticles may be a promising adjuvant to the most current approaches aiming at rheumatoid arthritis therapy.

Declaration of competing interest

There are no conflicts of interest to declare.

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