



Protective effect of a new hyaluronic acid -carnosine conjugate on the modulation of the inflammatory response in mice subjected to collagen-induced arthritis

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ABSTRACT

Several studies demonstrated the pharmacological actions of carnosine as well as hyaluronic acid (HA) during joint inflammation. In this study, the aim was to investigate the protective effect of a new HA-Carnosine conjugate (FidHycarn) on the modulation of the inflammatory response in mice subjected to collagen-induced arthritis (CIA). CIA was induced by two intradermal injections of 100 µl of an emulsion of collagen (CII) and complete Freund's adjuvant (CFA) at the base of the tail on day 0 and 21. At 35 days post CIA induction, the animals were sacrificed.

The injection caused erythema and edema in the hind paws, histological alterations with erosion of the joint space as well as behavioral changes. Oral treatment with FidHycarn starting at the onset of arthritis (day 25) and continuing for 10 days, improved behavioral deficits as well as decreased histological and radiographic alterations. The degree of oxidative damage evaluated by inducible nitric oxide synthase (iNOS), nitrotyrosine, poly-ADP-ribose (PAR) expressions and malondialdehyde (MDA) levels, was also significantly reduced in Carnosine + HA association and FidHycarn treated mice. Moreover, the levels of proinflammatory cytokines and chemokines and cyclo-oxygenase COX-2 enzyme were also more significantly reduced by Carnosine + HA and FidHycarn compared to carnosine alone. However, interestingly, in some cases, the effects of FidHycarn were more important than Carnosine + HA association and not statistically different to methotrexate (MTX) used as positive control.

Thus, the conjugation of Carnosine with HA (FidHycarn) could represent an interesting therapeutic strategy to combat arthritis disorders.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that causes

pain and inflammation in the joints with marked destruction of articular cartilage and bone erosion. Type II collagen-induced arthritis (CIA) in mice is an experimental model of RA, with characteristics of

Abbreviations: Carn, Carnosine; CFA, Complete Freund adjuvant; CIA, Type II collagen-induced arthritis; CII, Collagen type II; IL-1β, Interleukin-1β; IL-6, Interleukin-6; COX-2, cyclo-oxygenase; DMARDs, disease-modifying anti-rheumatic drugs; HA, hyaluronic acid; FidHycarn, HA conjugated with CARN; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; MPO, Myeloperoxidase; MTX, Methotrexate; PAR, poly-ADP-ribose; RA, Rheumatoid arthritis; ROS, Reactive oxygen species; SF, synovial fluid; TNF-α, Tumor necrosis factor-α

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cellular and humoral immunity typical of human RA. The pathogenesis of CIA is characterized by the response of the host to type II collagen and the subsequent generation of autoantibodies [1]. Furthermore, the recruitment of neutrophils, macrophages and lymphocytes in the joints with subsequent pannus growth are important signs of the pathogenesis of both the CIA and RA. In addition, the production of interleukin (IL)-8, MIP-1 α , MIP-1 β and RANTES with chemotatic action towards lymphocytes was also demonstrated [2]. In this regard, interleukin-1 (IL-1 β) is primarily implicated in the onset and development of the disease [3,4]. Oxidative stress plays a fundamental role in cartilage degradation during RA. In articular chondrocytes, reactive oxygen species (ROS) are IL-1 β signaling intermediates [5].

To date, treatments for RA disorder are represented by disease-modifying anti-rheumatic drugs (DMARDs) and biological drugs with an immunomodulatory action [6], inhibitors of cytokine and endothelial cell proliferation [7]. However, these composites have important serious side effects and extensive toxicity differences among DMARDs exist [8]. Methotrexate (MTX), a classic DMARD, is a common immunosuppressant representative in the treatment of RA patients. MTX involves metabolic changes with increased extracellular adenosine and suppression of immune cell function [9]. For this reason, the toxicity is the main reason for methotrexate inconstancy [10]. In this regard, new therapeutic strategies are important. Hyaluronan (HA) is an important element of synovial fluid (SF) needed in joint function [11]. Several studies have shown that in outbreaks of inflammation, HA is depolymerized through ROS production and degradation by enzymes such as hyaluronidase, β -glucuronidase, chondroitinase and hexosaminidase [12]. The minor HA fragments indicate a possible endogenous danger indicator that determines the activation of innate and acquired immunity. However, since hyaluronidase is not present in SF, it is conceivable that ROS are also involved during HA catabolism in a damaged joint. Transition metals, for example iron or copper, also play a central responsibility in the oxidative catabolism of HA. The primary purposes of HA are size-specific, while the major portion of hyaluronan have immunosuppressive and anti-angiogenic activity. The intermediate-sized HA portions are inflammatory, immunostimulatory and highly angiogenic [13]. Therefore, substances that inhibit the degradation of HA could be anti-inflammatory and anti-angiogenic. However, additionally, previous studies also reported a marked antioxidant action of HA [14–16]. In this regard, it was also shown the efficacy of oral administration of HA powder on knee osteoarthritis (OA) [17]. Carnosine, β -alanine-histidine (CARN) is a natural dipeptide with several physiological and protective properties: pH buffering, ROS, RNS and RCS scavenging, anti-glycating, heavy metal chelating, neuroprotective and catabolic treatment [18–22]. Indeed, CARN has been found to exert a protective action on the HA degradation due to ROS ($\cdot$ OH and/or peroxyl-type radicals) in the *in vitro* assays; this inhibitory activity results in an effective comparison to methotrexate. Furthermore, CARN proved its antioxidant properties also *in vivo* by decreasing the systemic markers (thiobarbituric acid reacting substances and malondialdehyde) of redox imbalance occurring in adjuvant arthritis. Furthermore, it exerted an anti-inflammatory action, demonstrated in the reduction of hind paw volume in arthritic animals [23]. Carnosine hydrolysis into its constituting amino acids occurs because of the activity of the carnosinases CNBP1 and CNBP2. CNBP1 specifically degrades both carnosine and homocarnosine (the GABA analogue of carnosine), while CNBP2 is a manganese-dependent cytosolic non-specific dipeptidase, ubiquitously expressed in human tissues and able to degrade carnosine and other dipeptides [24–26].

The synthesis of carnosine derivatives obtained by conjugation with different carbohydrate moieties allowed for the inhibition of carnosinase activities, paving the way also for the oral use of carnosine [27–29].

Recently, the synthesis of CARN conjugated with hyaluronic acid has been reported with significant antioxidant activities; in this new molecular entity HA protects CARN from carnosine degradative attack

while CARN acts as a scavenger against ROS insults that can alter the molecular features of HA [30].

Thus, based on these findings, the aim of this work was to investigate the synergistic effect of a new Carn conjugated with HA (FidHycarn) obtained by the covalent conjugation of CARN to HA with biopharmacological effects of both compounds, on the modulation of the inflammatory response in mice subjected to CIA.

2. Material and methods

2.1. Animals

DBA/1 J male mice (9 weeks; 27–30 g, C57BL/6J), were used for this experiment. Water and food were accessible *ad libitum*. This study was authorized by the University of Messina Review Board for the care of animals. Animal experiments complied with ARRIVE guidelines. Animal care was in conformity with the new legislation for the safety of animals used for scientific experiments (Directive 2010/63/EU).

2.2. CIA induction

Type II chicken collagen (CII) was mixed in 0,01 M acetic acid at a concentration of 2 mg/ml overnight at 4 °C and saved at - 70 °C. Complete Freund's adjuvant (CFA) was mixed with *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg/ml. An emulsion of CII with an equal volume of CFA was created before injection. Mice were injected intradermally at the base of the tail with 100 μ l of emulsion (containing 100 μ g of CII) on day 1. A second CII injection in CFA was done in mice on day 21 [31].

2.3. Experimental groups

Mice were divided into the following experimental groups:

- Sham – Vehicle; mice subjected to an intradermal injection at the base of the tail of 100 μ l of 0,01 M acetic acid instead of the emulsion containing 100 μ g of CII, were orally administered with vehicle (distilled water) for FIDHYCARN every 24 h, starting from day 25 to day 35 (n = 20).
- Sham – FIDHYCARN; mice subjected to an intradermal injection at the base of the tail of 100 μ l of 0,01 M acetic acid instead of the emulsion containing 100 μ g of CII, were administered with FIDHYCARN (88,5 mg/kg, orally) every 24 h, starting from day 25 to day 35 (n = 20).
- CIA-Control; mice were subjected to CIA as described above. Mice were treated orally with distilled water (vehicle for FIDHYCARN) every 24 h, starting from day 25 to day 35 (n = 20).
- CIA + Carn; mice subjected to CIA were administered with Carn (17,6 mg/kg, orally) every 24 h, starting from day 25 to day 35 (n = 20).
- CIA + Carn; mice subjected to CIA were administered with Carn (13,2 mg/kg, orally) every 24 h, starting from day 25 to day 35 (n = 20).
- CIA + Carn; mice subjected to CIA were administered with Carn (8,8 mg/kg, orally) every 24 h, starting from day 25 to day 35 (n = 20).
- CIA + Carn; mice subjected to CIA were administered with Carn (13,2 mg/kg, orally) twice a day starting from day 25 to day 35 (n = 20).
- CIA – Carn + HA; mice subjected to CIA were administered with Carn + HA (17,6 mg/kg of Carn and 70,9 mg/kg of HA, orally) every 24 h, starting from day 25 to day 35 (n = 20).
- CIA – Carn + HA; mice subjected to CIA were administered with Carn + HA 13,2 mg/kg of Carn and 53,17 mg/kg of HA, orally every 24 h, starting from day 25 to day 35 n = 20.
- CIA – Carn + HA; mice subjected to CIA were administered with Carn + HA 8,8 mg/kg of Carn and 35,45 mg/kg of HA, orally every 24 h, starting from day 25 to day 35 n = 20.

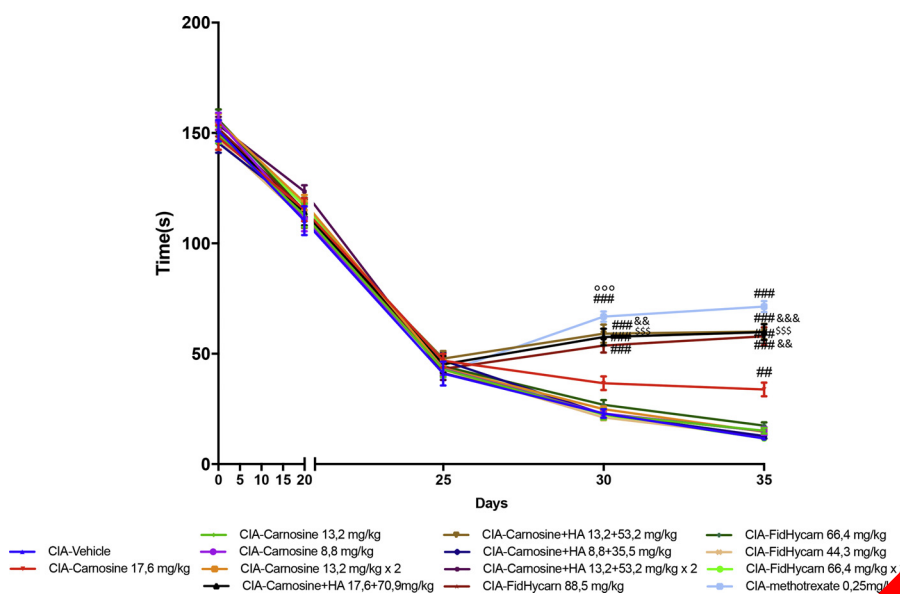


Fig. 1. The effects of Carn+HA association and FIDHYCARN on motility during CIA. Locomotor activities on the rotarod are ameliorated in CIA treated with Carn at 17,6 mg/kg and in particular Carn+HA association (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) and FIDHYCARN at higher dose of 88,5 mg/kg than in CIA + vehicle mice. Figures are representative of all the animals in each group. Methotrexate is used as positive control. Values are given as mean $\pm$ SEM of 20 animals for each group. ## $p < 0,01$ vs CIA, ### $p < 0,001$ vs CIA, °°° $p < 0,001$ vs CIA, && $p < 0,01$ vs CIA, &&& $p < 0,001$ vs Carn 17,6 mg/kg, \$\$\$ $p < 0,001$ vs Carn 13,2 mg/kg, \$\$ $p < 0,01$ and \$ $p < 0,001$ vs Carn 17,6 mg/kg.

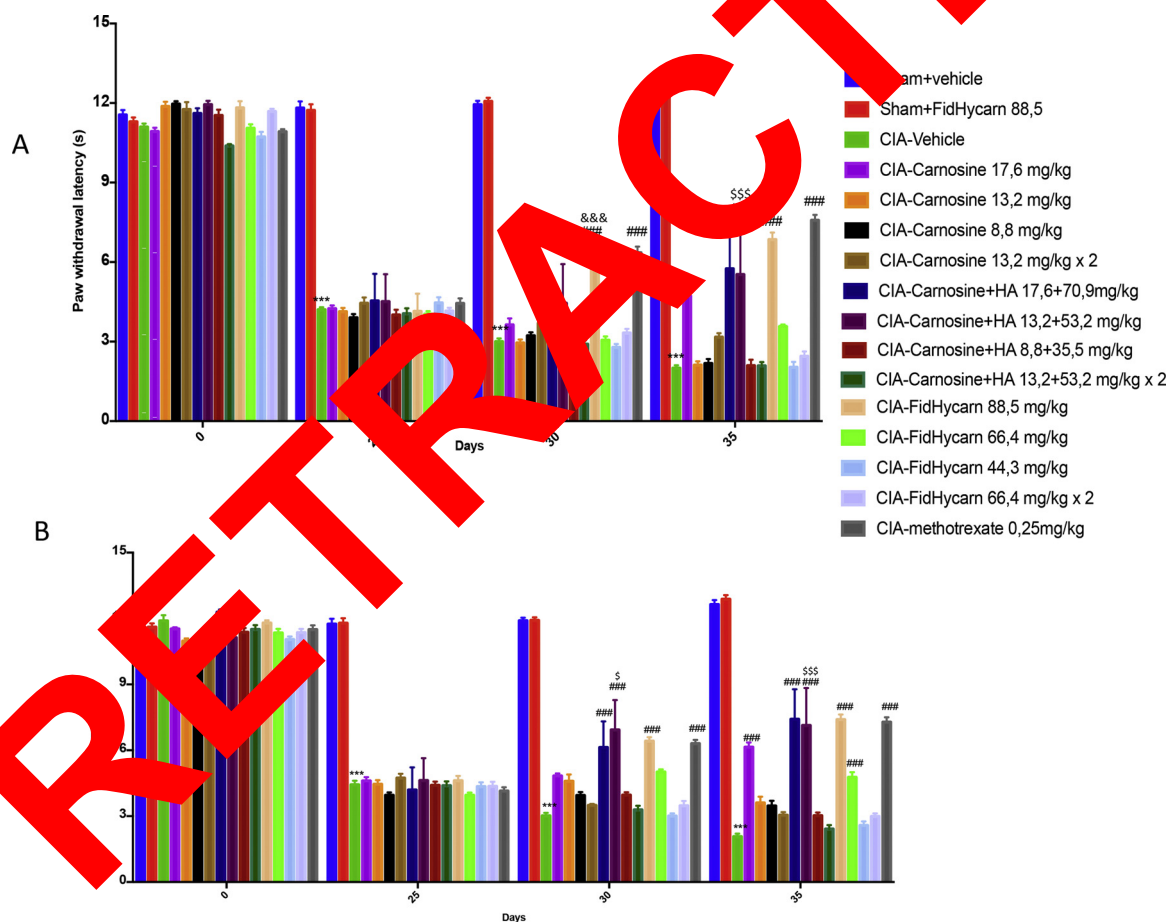


Fig. 2. The effects of Carn + HA association and FIDHYCARN on pain during CIA. No alteration was found in sham animals (A, B). In addition, CIA mice presented increased thermal hyperalgesia (A) and pain sensitivity (B) compared to sham. Carnosine at 17,6 mg/kg and in particular Carn + HA association (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) and FIDHYCARN treatment (88,5 mg/kg) decreased thermal hyperalgesia and pain sensitivity in a more significant way (A, B). Methotrexate is used as positive control. Figures are representative of all the animals in each group. Values are given as mean $\pm$ SEM of 20 animals for each group. *** $p < 0,001$ vs sham-control, ## $p < 0,01$ vs CIA, ### $p < 0,001$ vs CIA, && $p < 0,001$ vs Carn 17,6 mg/kg, \$ $p < 0,05$ and \$\$\$ $p < 0,001$ vs Carn 13,2 mg/kg.

· CIA – Carn + HA; mice subjected to CIA were administered with Carn + HA 13,2 mg/kg of Carn and 53,17 mg/kg of HA, orally twice a day, starting from day 25 to day 35 $n = 20$.

· CIA – FIDHYCARN; mice subjected to CIA were administered with

FIDHYCARN (88,5 mg/kg, orally) every 24 h, starting from day 25 to day 35 ($n = 20$).

· CIA – FIDHYCARN; mice subjected to CIA were administered with FIDHYCARN 66,37 mg/kg, orally every 24 h, starting from day 25 to

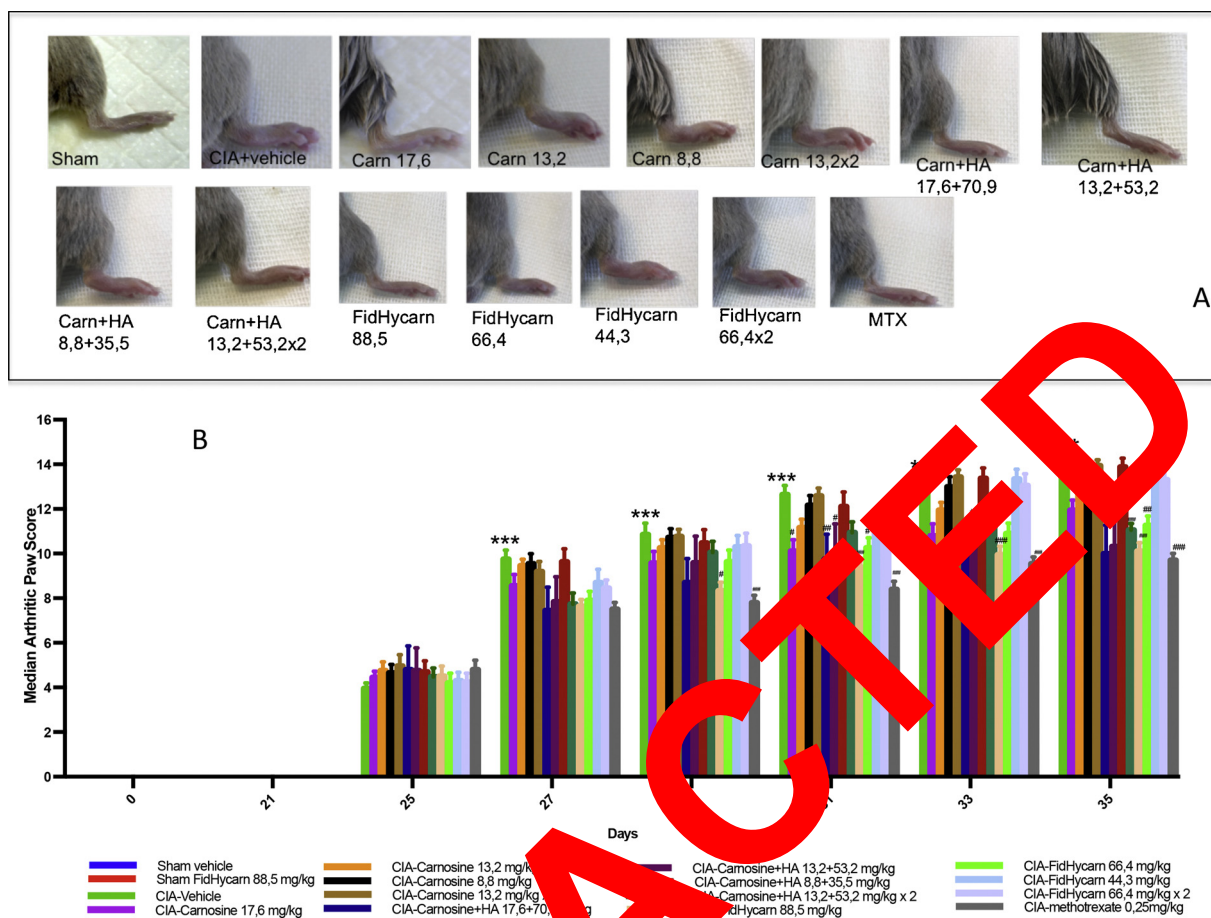


Fig. 3. The effects of Carn + HA association and FIDHYCARN on macroscopic alterations. Clinical signs like periarticular erythema and edema were found in CIA + vehicle mice A. Carnosine at 17,6 mg/kg, and in particular Carn + HA combination 17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg and FIDHYCARN at higher dose of 88,5 mg/kg showed a reduction of clinical signs of arthritis more than others. No clinical signs were found in sham animals A. Methotrexate is used as positive control. Figures are representative of all the animals of each group. Median arthritic paw score is showed in B. Values are expressed as mean $\pm$ SEM of 20 animals for each group. ***p < 0,001 vs sham-control, #p < 0,05 vs CIA, ##p < 0,01 vs CIA, ###p < 0,001 vs CIA.

day 35 n = 20.

· CIA – FIDHYCARN; mice subjected to CIA were administered with FIDHYCARN 44,25 mg/kg, orally every 24 h, starting from day 25 to day 35 n = 20.

· CIA – FIDHYCARN; mice subjected to CIA were administered with FIDHYCARN 66,37 mg/kg, orally once a day, starting from day 25 to day 35 n = 20.

· CIA - Methotrexate; mice subjected to CIA were administered with Methotrexate (G860, 0,25 mg/kg, orally) every 24 h, starting from day 25 to day 35 (n = 20).

No statistical differences were observed in all sham groups, for this reason only the results regarding Carn + vehicle group were showed in histological and immunohistochemical analyses.

2.4. Synthesis of FIDHYCARN

The synthesis of FIDHYCARN is described in the patent WO2019069258 [32]. Briefly, the first step is the synthesis of carnosine methyl ester. 1,5 g of carnosine was treated under stirring in a 250 ml flask with 50 ml of an acetyl chloride solution in anhydrous methanol (pre-mixed) in a 1:20 ratio (v/v) and successively about 90 % of the solvent was removed by evaporation. 20 ml of anhydrous methanol was then added to the reaction residue and again 90 % of the solvent was removed by evaporation. The operation was repeated until all HCL (which was formed during the reaction) had been removed; the product was then brought to dryness under vacuum. Following this step, 1,1 g of about 700 kDa hyaluronic acid were introduced in a reactor

with 80 ml of a mixture of H₂O and DMSO. Successively, a solution of H₂O and DMSO containing tris 2-(2-methoxyethoxy) ethyl amine, 3-hydroxy 1,2,3 benzotriazin 4 (3 H) one and N (3 dimethylaminopropyl) N ethylcarbodiimide hydro-chloride was added. Successively, 365 mg of carnosine methyl ester in DMSO were added. The product was precipitated with the addition of ethanol. The final precipitate was dissolved in water and subsequently lyophilized.

100 mg of the FIDHYCARN batch used in this in vivo study are constituted by 19,89 mg of carnosine and 80,11 mg of hyaluronic acid.

2.5. Clinical severity of CIA

The progress of arthritis in mice in all experimental groups was assessed daily beginning from day 20 after the first intradermal injection by using a macroscopic scoring system [33]. Arthritic index for each mouse was calculated by adding the four scores of individual paws. Clinical severity was also determined by quantitating the change in the paw volume using plethysmometry (model 7140; Ugo Basile).

2.6. Behavioral function after CIA induction

Rotarod: Locomotor abilities were assessed using a protocol previously employed [34]. DBA/1 J mice were given three days of training on the rotarod before disease induction. Trials were conducted, starting the 20 day after CIA induction, every five day until day 35. Each mouse was given three trials, after which the average time of a mouse remained on the rotating beam was calculated.

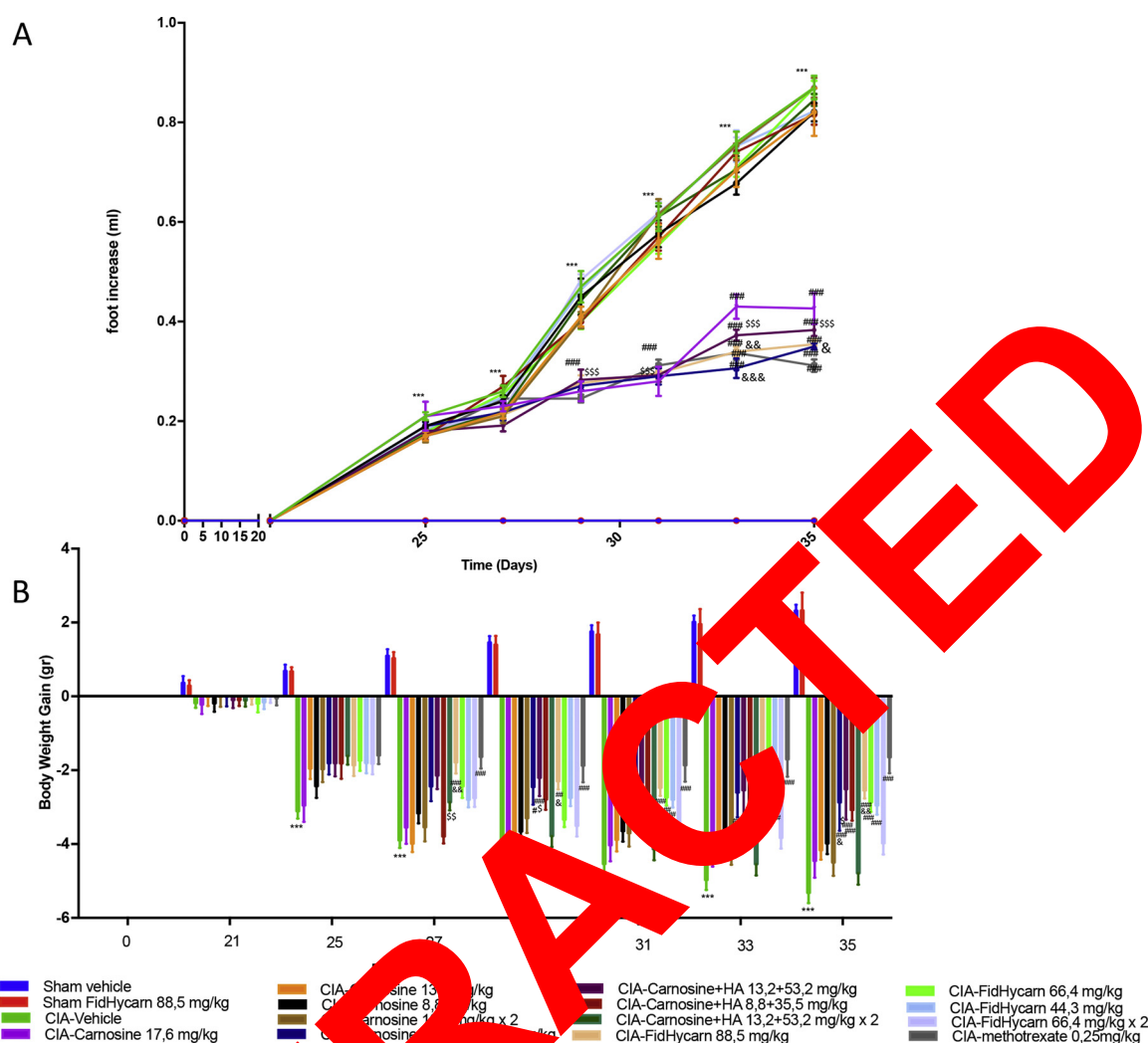


Fig. 4. The effects of Carn + HA association and FidHycarn on body weight and foot increase. CIA mice presented increased paw edema A and reduced body weight B compared to sham. Carnosine and particularly the association of Carn + HA and FidHycarn decreased the loss in body weight and paw edema A, B. Methotrexate is used as positive control. Values are expressed as mean $\pm$ SEM of 20 animals for each group. *** $p < 0,001$ vs sham-control, # $p < 0,05$ vs CIA, ## $p < 0,01$ vs CIA, ### $p < 0,001$ vs CIA, &&& $p < 0,001$, && $p < 0,01$, & $p < 0,05$ versus Carn 17,6 mg/kg, $^s p < 0,05$, $^{ss} p < 0,01$ and $^{sss} p < 0,001$ versus Carn 13,2 mg/kg.

Pain sensitivity testing. Hotplate testing was used to evaluate pain sensitivity as previously described [35].

Thermal hyperalgesia: Thermal nociceptive responses to heat was determined by the paw withdrawal latency using a Basile Plantar Test [36] (Ugo Basile, Comerio, Italy). For paw withdrawal latencies were taken on day 0 before CIA induction (baseline) and subsequently on day 25, 30 and 35 of experimental period. A significant ($P < 0,05$) reduction in paw-withdrawal latency over time is characterized as thermal hyperalgesia.

2.7. Histological analysis

On day 35, animals were sacrificed and paws and knees were collected and fixed in 10 % formalin. The paws were then cropped, placed in decalcifying solution for 24 h, implanted in paraffin, sectioned at 5 μ m, stained with hematoxylin/eosin and studied using light microscopy (Dialux 22 Leitz). The following morphological criteria were considered: 0 = no damage; 1 = edema; 2 = inflammatory cell presence; 3 = bone resorption [37].

2.8. Immunohistochemical localization of nitrotyrosine, poly ADP ribose (PAR), iNOS, and COX-2

Immunohistochemical analyses were performed as previously described [31]. Sections were incubated overnight with 1) anti-rabbit polyclonal antibody directed at iNOS (1:1000 in PBS, v/v) (DBA, Milan, Italy) or 2) anti-COX-2 goat polyclonal antibody (1:500 in PBS, v/v) or 3) anti-nitrotyrosine rabbit polyclonal antibody (1:1000 in PBS, v/v) or 4) with anti-PAR goat polyclonal antibody rat (1:500 in PBS, v/v). As a general procedure, the digital images were opened in ImageJ, followed by deconvolution using the color deconvolution plug-in. When the IHC profiler plug-in is selected, it automatically plots a histogram profile of the deconvoluted DAB image, and a corresponding scoring log is displayed [38]. The histogram profile corresponds to the positive pixel intensity value obtained from the computer program [39].

2.9. Radiography

Radiographic analysis was performed by X-Ray Bruker FX Pro instrument (Milan, Italy). The following radiographic criteria from hind limbs will be considered: score 0, no bone damage; score 1, tissue swelling and edema; score 2, joint erosion; 3, bone erosion and

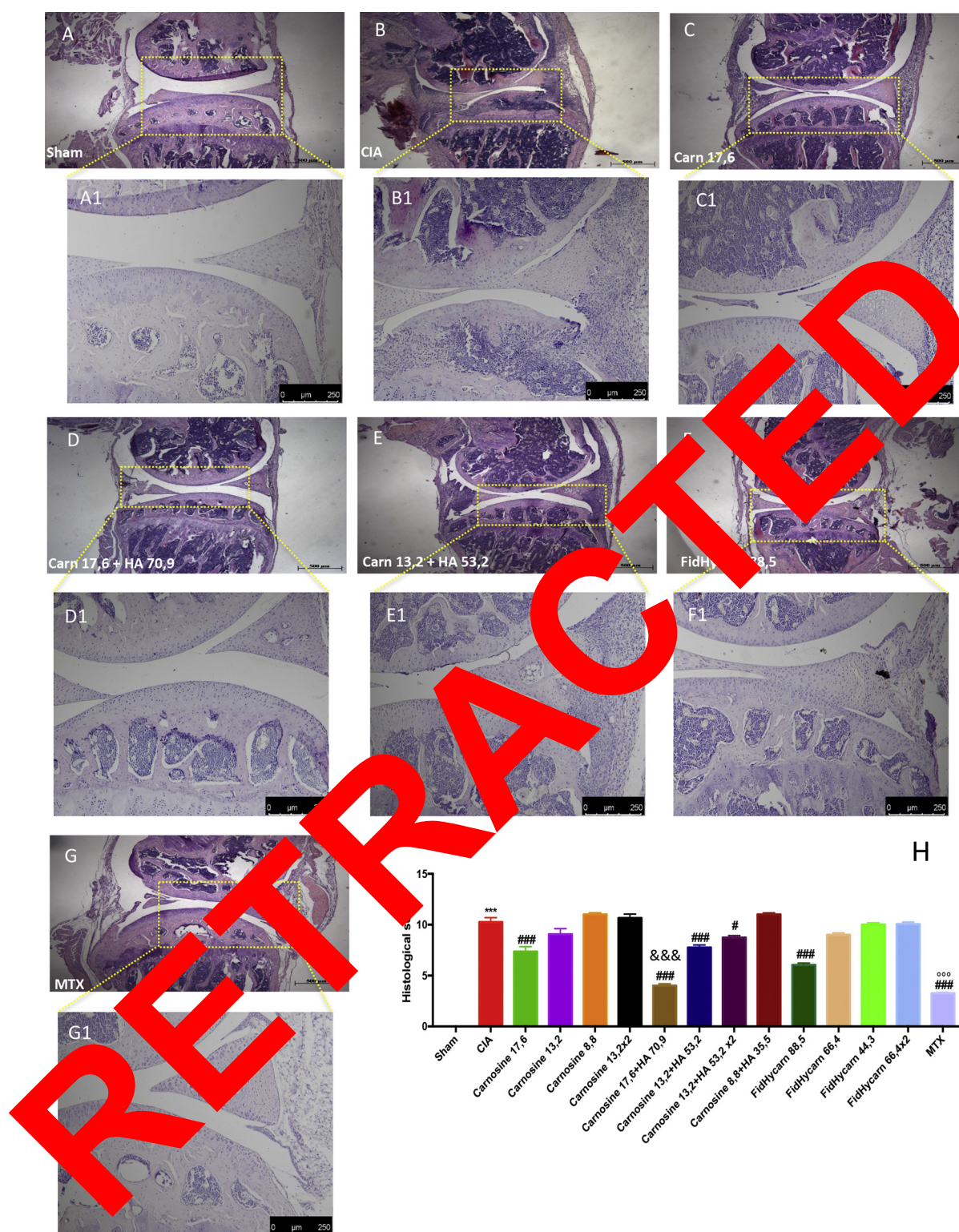


Fig. 5. The effects of Carn + HA association and FidHycarn on histological damage. The histological evaluation by hematoxylin/eosin-staining of joint sections showed inflammatory cell infiltration and bone erosion in CIA-control mice (B, B1) compared to sham animals (A, A1). Ameliorated histological alterations were observed in the sections from CIA-Carnosine (17,6 mg/kg) and more significantly in Carn + HA association (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) (C, C1, D, D1, E, E1) and FidHycarn (88,5 mg/kg) treated mice (F, F1). Methotrexate is a positive control (G). Histological score is shown in H. Figures are representative of all the animals in each group. Values are given as mean $\pm$ SEM of 20 animals for each group. ***p < 0.001 vs sham-control, #p < 0,05 vs CIA, ###p < 0,001 vs CIA, °°°p < 0,001 FidHycarn 88,5 mg/kg versus methotrexate, &&&p < 0,001 versus Carn 17,6 mg/kg.

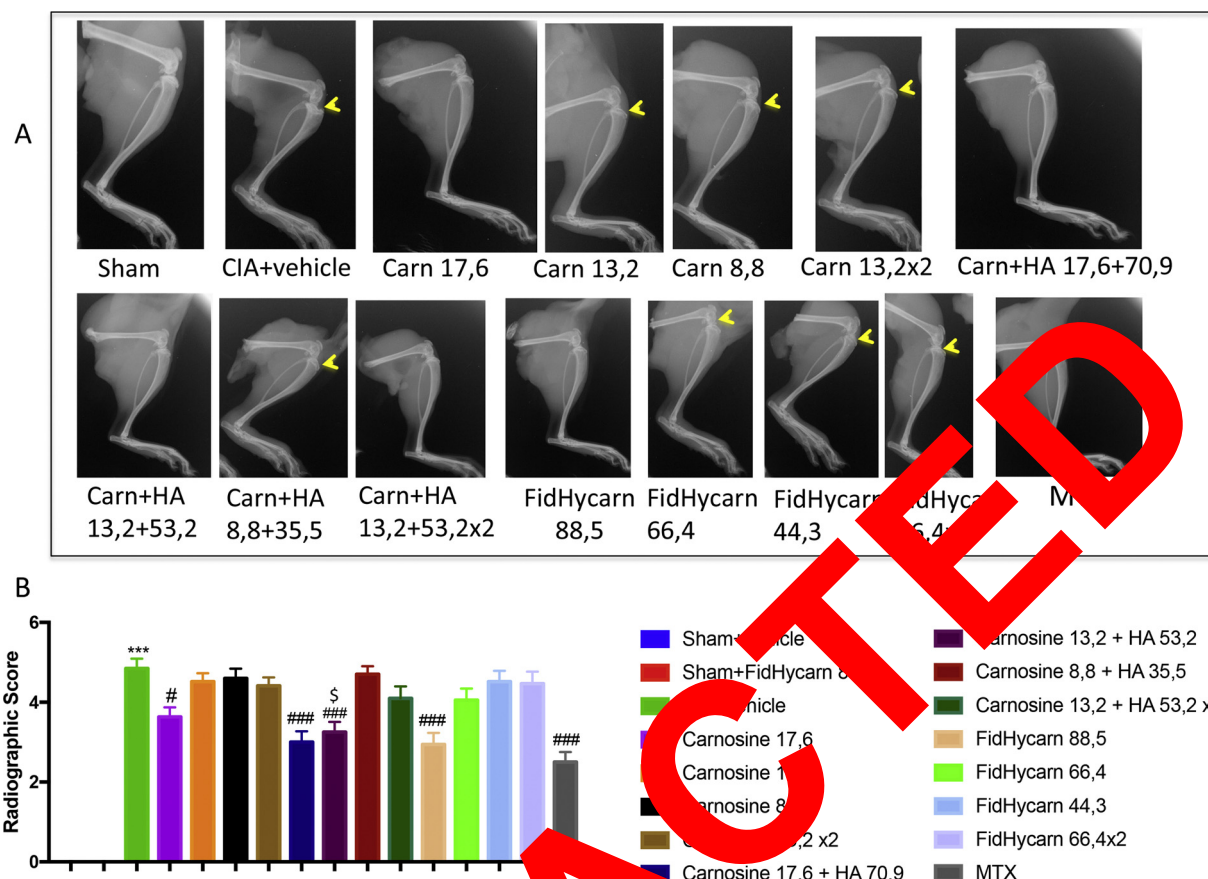


Fig. 6. The effects of Carn + HA association and FidHycarn on radiographic damage. The design of pathology in the femoral growth plate and the tibiotarsal joints of normal mice was observed. A. Hindpaws from CIA + vehicle mice presented bone resorption in the femoral growth plate and in the tibiotarsal joints. A. Mice treated with carnosine 17,6 mg/kg and in particular Carn + HA association 17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg and higher dose of FidHycarn 88,5 mg/kg have shown less bone erosion in the femoral growth plate as well as in the tibiotarsal joints. Radiographic score is shown in B. Figures are representative of all the animals in each group. Values are expressed as mean $\pm$ SEM of 6 animals for each group. ***p < 0,001 vs sham-control, #p < 0,05 vs CIA, ###p < 0,001 vs CIA, \$p < 0,05 versus Carn at 13,2 mg/kg.

osteophyte formation [37].

2.10. Measurement of cytokines

Tumor Necrosis Factor (TNF- α), interleukin (IL)-6 and IL-1 β levels were evaluated in the plasma from CIA and sham mice. The assay was carried out using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, San Diego, Italy) [40].

2.11. Measurement of chemokines

Levels of chemokines MIP-1 α and MIP-2 were measured in the aqueous joint extract [31].

2.12. Thiobarbituric acid-reactant substances measurement (MDA levels)

Thiobarbituric acid-reactant substances measurement, which is considered a good indicator of lipid peroxidation, was determined as previously indicated [41].

2.13. Myeloperoxidase (MPO) assay

Neutrophil infiltration to the inflamed joints was indirectly determined using an MPO assay, as previously described for neutrophil elicitation [42].

2.14. Materials

All drugs were kindly offered from FIDIA Farmaceutici spa. Other compounds were acquired from Sigma-Aldrich Company (Milan, Italy). All chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9 % NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.) or 10 % ethanol (Sigma-Aldrich).

2.15. Data analysis

All values in the figures and text were expressed as mean standard error (s.e.m.) of the mean of n observations. For the in vivo studies n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown (1 figure for each experimental group) is representative of all joint tissue sections collected from all the animals in each group. Data sets were examined by one- or two-way analysis of variance followed by Bonferroni test for multiple comparisons. A p-value of less than 0,05 was considered significant.

3. Results

3.1. Effects of Carn, Carn + HA association and FidHycarn on behavioral function after CIA induction

First, we evaluated how prolonged daily treatments with Carn, Carn

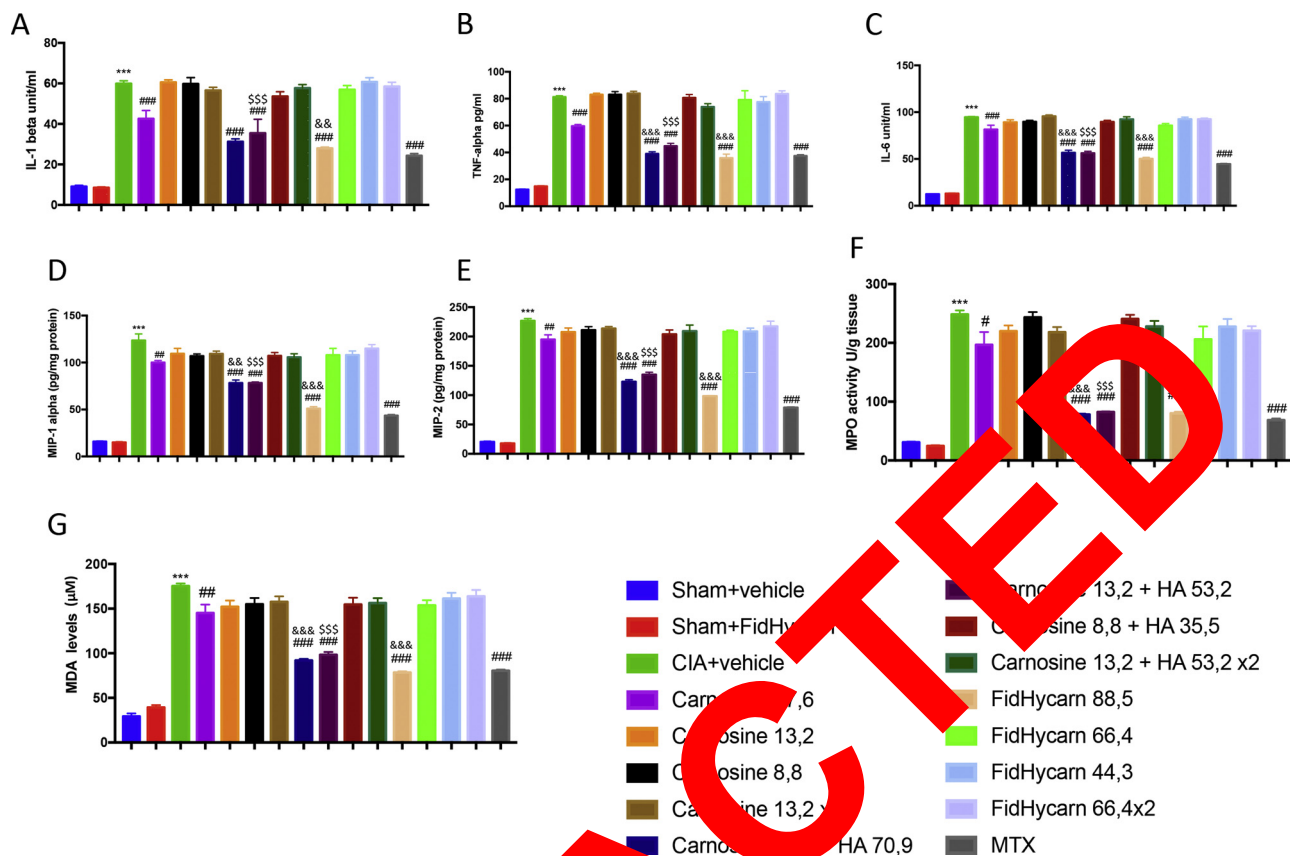


Fig. 7. The effects of Carn + HA association and FidHycarn on cytokine production, neutrophil infiltration and MDA levels. IL-1 β A, TNF- α B, IL-6 C, MIP-1 α D, MIP-2 E levels and MPO activity F and MDA levels G were shown. Data are given as mean $\pm$ SEM of 20 animals for each group. ***p < 0,001 vs sham-control, ##p < 0,01 vs CIA, ###p < 0,001 vs CIA, &&&p < 0,001, &p < 0,05, and \$\$\$p < 0,001 versus Carn 13,2 mg/kg.

+ HA and FidHycarn ameliorated the locomotor disability, evaluated by performance on a non-accelerating rotarod. The effect of Carnosine at higher dose of 17,6 mg/kg and in particular Carnosine + HA association (at doses of 17,6 + 70,9 and 13,2 + 53,2 mg/kg) and FidHycarn at higher dose of 88,5 mg/kg significantly reduced the motor impairment in CIA mice compared to vehicle group (Fig. 1). The lower doses of Carn (13,2 and 8,8 mg/kg), Carn + HA (13,2 + 35,5 mg/kg) and FidHycarn (66,4 and 44,3 mg/kg) have no the same protective effects (Fig. 1). The effect of FidHycarn at 88,5 mg/kg was statistically different compared to methotrexate at day 35 post CIA (Fig. 1) and not at the latest time point at 25 days post CIA. Furthermore, at day 25 after CIA induction, mice showed a significant reduction in hind paw withdrawal latency to a maximum hypersensitive response observed between day 30–35 post CIA in CIA-control mice. (Fig. 2A). Daily oral treatments with Carnosine 17,6 mg/kg reduced significantly CIA-induced thermal hyperalgesia at only 35 day post CIA, whereas the lower doses of Carn (13,2 and 8,8 mg/kg) were not able to reduce it. In addition, Carnosine + HA (17,6 + 70,9 and 13,2 + 53,2 mg/kg) and FidHycarn at 88,5 mg/kg (Fig. 2 A) reduced more significantly thermal hyperalgesia compared to others with no statistical difference observed between FidHycarn at 88,5 mg/kg and methotrexate groups (Fig. 2 A). The lower doses of Carn + HA and FidHycarn have no the same protective effect (Fig. 2 A). Then, the effect of FidHycarn on pain sensitivity was tested by subjecting mice to hotplate testing and recording latency to a response. At days 25 after CIA induction, CIA + vehicle mice exhibited increased pain sensitivity compared to sham. Moreover, between day 30–35 post immunization, the treatment of Carnosine 17,6 mg/kg and in particular Carnosine + HA (17,6 + 70,9 and 13,2 + 53,2 mg/kg) and FidHycarn at 88,5 and 66,4 mg/kg reduced pain sensitivity

compared to all others (Fig. 2 B). No statistical difference was observed between FidHycarn at 88,5 mg/kg and methotrexate groups (Fig. 2 B). The amelioration of behavior was more evident in Carn + HA (17,6 + 70,9 and 13,2 + 53,2 mg/kg) versus Carn alone at 17,6 mg/kg or 13,2 mg/kg. An important statistical difference was also observed between Carn at 17,6 mg/kg and Fidhycarn at 88,5 mg/kg groups.

3.2. Effects of Carn, Carn + HA association and FidHycarn on clinical signs and body weight increase during CIA

First macroscopic signs (periarticular erythema and edema) of CIA in mice immunized with CII were observed in hindpaws between 24 and 26 days after CIA induction in all experimental groups (Fig. 3 A and see 3B). The treatments with Carn at 17,6 mg/kg, Carn + HA (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) and FidHycarn in particular at 88,5 mg/kg significantly reduced the development of joint inflammation with no statistical difference with methotrexate (MTX) (Fig. 3 A and see 3B). No clinical signs of CIA were observed in the paws of sham groups (Fig. 3 A and see 3B).

In addition, the Fig. 4A demonstrated a time-dependent increase in paw volume (each value are the mean values of both hindpaws). The oral treatments with Carn at 17,6 mg/kg, Carn + HA (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) and FidHycarn in particular at 88,5 mg/kg reduced foot increase. No statistical difference was observed between FidHycarn at 88,5 mg/kg and methotrexate groups. No increases in hindpaw volume were observed in sham groups (Fig. 4A).

The gain in body weight was similar in sham and in CIA mice in the first week. From day 25, CII-injected mice gained less weight than the sham animals, until day 35. The treatments with Carn + HA and FidHycarn were able to increase body weight in a significant way

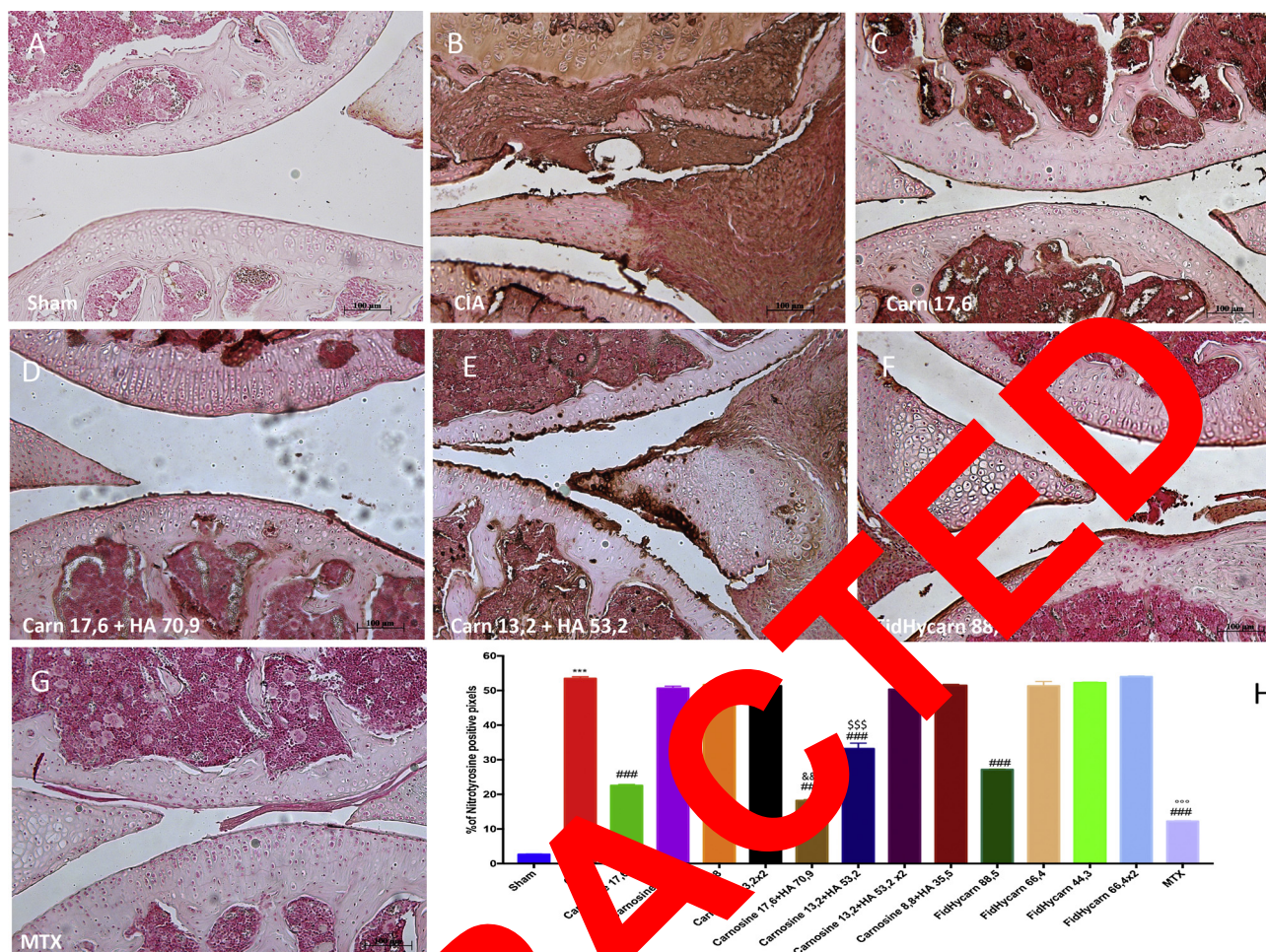


Fig. 8. The effects of Carn + HA association and FidHycarn on nitrotyrosine expression. Immunohistochemistry for nitrotyrosine in joint tissues respectively A sham group, B CIA + vehicle group, C, D, E CIA + Carn 13.2 mg/kg, F CIA + Carn 17.6 mg/kg, G CIA + FidHycarn treatment group, H CIA + methotrexate. The results are expressed as % of positive pixels H. Figures are representative of at least three independent experiments. Values are means $\pm$ SEM of 20 animals for each group; ***P < 0,001 vs sham, #P < 0,05 vs CIA, ###P < 0,001 vs CIA, \$\$\$P < 0,001 vs Carn 13.2 mg/kg, ****P < 0,001 vs FidHycarn 88,5 mg/kg versus methotrexate, &&&p < 0,001 versus Carn 17,6 mg/kg, \$\$\$p < 0,001 versus Carn 13.2 mg/kg.

compared to others. No statistical difference was observed between FidHycarn at 88,5 mg/kg and methotrexate groups (Fig. 4B). The amelioration of clinical signs was more evident in Carn+HA (17,6 + 70,9 and 13,2 + 32,2 mg/kg) versus Carn alone at 17,6 mg/kg or 13,2 mg/kg. An important statistical difference was also observed between Carn at 17,6 mg/kg and FidHycarn at 88,5 mg/kg groups.

3.3. Effect of Carn, Carn + HA association and FidHycarn on histological analysis during CIA

At day 30, the histological evaluation of the paws from CIA + vehicle mice revealed signs of severe arthritis, with bone erosion and necrosis (Fig. 5 B, B1 and see histological score 5 H) compared to sham (Fig. 5 A, A1 and see histological score 5 H). The oral treatment with Carn at 17,6 mg/kg was able to reduce histological alterations compared to vehicle group (Fig. 5 C, C1 and see histological score 5 H). However, Carn at lower doses of 13,2 (also double administration) and 8,8 mg/kg were not able to have a protective effect (data not shown, see histological score 5 H). In addition, Carn + HA (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) association was also able to markedly reduce joint inflammation (Fig. 5 D, D1, E, E1 and see H), where as Carn + HA association at lower doses of 8,8 + 35,5 mg/kg was not (data not shown, see histological score 5 H). The oral treatment of FidHycarn at higher dose of 88,5 mg/kg more significantly reduced the bone erosion and the necrosis of joint from CIA mice respect to lower doses (66,4 and

44,3 mg/kg) (Fig. 5 F, F1 and see histological score 5 H). Anyway, there was a statistical difference between FidHycarn and MTX (positive control) (Fig. 5 G, G1 and see histological score 5 H).

3.4. Effects of Carn, Carn + HA association and FidHycarn on radiographic analysis during CIA

Radiographic analysis of knee joint from CIA + vehicle mice at 35 days after CII injection also revealed bone erosion (Fig. 6 A and see radiographic score B). The oral treatment with Carn at higher dose of 17,6 mg/kg (Fig. 6 A and see B) was able to ameliorate radiographic alterations respect to lower doses (13,2 and 8,8 mg/kg) (Fig. 6 A and see B). The double administration of Carn at 13,2 mg/kg has the same effect of single administration (Fig. 6 A and see B). In addition, the association of Carnosine+HA (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) was able to significantly reduced joint damage (Fig. 6 A and see B). The administration of Carn+HA at lower doses of 8,8 + 35,5 mg/kg was not able to have the same protective effect (Fig. 6 A and see B). In particular, FidHycarn molecule at higher dose of 88,5 mg/kg more significantly reduced bone erosion (Fig. 6 A and see B) in comparison to lower doses (single or double administration at 66,4 mg/kg and 44,3 mg/kg) (Fig. 6 A and see B). No statistical difference was observed between FidHycarn at 88,5 mg/kg and MTX groups (Fig. 6 A and see B). No evidence of bone resorption was found in sham mice. (Fig. 6 A and see B).

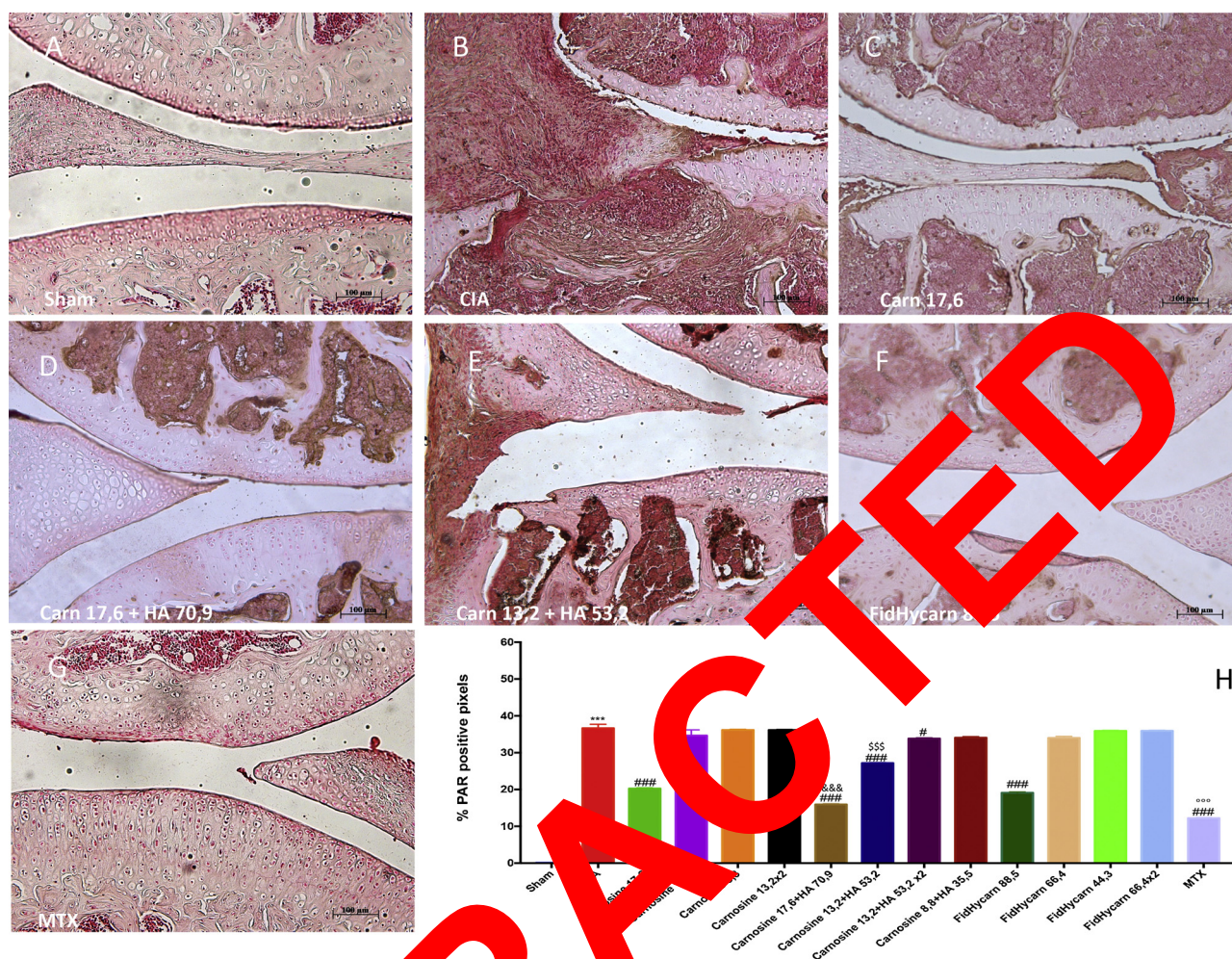


Fig. 9. The effects of Carn + HA association and FidHycarn on PAR expression. Immunohistochemistry for PAR in joint tissues respectively A sham group, B CIA + vehicle group, C, D, E CIA + Carn + HA association, F Carn + HA association treatment group, G CIA + methotrexate. The results are expressed as % of positive pixels H. Figures are representative of at least three independent experiments. Values are means $\pm$ SEM of 20 animals for each group; *** $p < 0.001$ vs sham, # $p < 0.05$ vs CIA, ### $p < 0.001$ vs CIA, &&& $p < 0.001$ vs Carn 17,6 mg/kg, &&& $p < 0.001$ vs Carn 13,2 mg/kg.

3.5. Effects of Carn, Carn + HA association and FidHycarn on proinflammatory cytokines and chemokines levels during CIA

In this study we also evaluated the levels of the proinflammatory cytokines TNF- α , IL-1 β , and chemokines MIP-1 α and MIP-2 production was found in CIA mice 35 days after immunization. Levels of TNF- α , IL-1 β and chemokines MIP-1 α and MIP-2 were reduced in CIA mice treated with Carnosine at higher dose of Carnosine 17,6 mg/kg, while the lower doses of Carnosine (13,2 and 8,8 mg/kg) were not able to reduce cytokines and chemokines levels (Fig. 7 A, B, C, D, E). In addition, Carnosine + HA (17,6 + 70,9 and 13,2 + 53,2 mg/kg) and FidHycarn at higher dose of 88,5 mg/kg were able to reduce cytokines and chemokines levels in a more significant way compared to others (Fig. 7 A, B, C, D, E). Moreover, the effects of FidHycarn at 88,5 mg/kg was not statistical different to methotrexate (Fig. 7 A, B, C, D, E).

The reduction on pro-inflammatory cytokines and chemokines levels was more evident in Carn + HA (17,6 + 70,9 and 13,2 + 53,2 mg/kg) versus Carn alone at 17,6 mg/kg or 13,2 mg/kg. An important statistical difference was also observed between Carn at 17,6 mg/kg and Fidhycarn at 88,5 mg/kg groups.

3.6. Effects of Carn, Carn + HA association and FidHycarn on MPO activity and MDA levels during CIA

MDA levels were also detected in the plasma as an indicator of lipid peroxidation, at 35 days after CIA induction. In addition, joint neutrophil infiltration was also performed by MPO activity. A significant increase of MPO and MDA levels were observed from mice subjected to CIA when compared to sham groups (Fig. 7 F, G). MPO and MDA levels were significantly reduced in mice treated daily with oral treatments of Carnosine 17,6 mg/kg while the lower doses of Carnosine (13,2 and 8,8 mg/kg) were not able to reduce these parameters levels (Fig. 7 F, G). In addition, Carnosine + HA associations (17,6 + 70,9 and 13,2 + 53,2 mg/kg) and FidHycarn at higher dose of 88,5 mg/kg were also able to reduce MPO and MDA levels in a more significant way (Fig. 7 F, G). The effect of FidHycarn at 88,5 mg/kg was not statistical different to methotrexate (Fig. 7 F, G). The reduction on MPO activity and MDA levels was more evident in Carn + HA (17,6 + 70,9 and 13,2 + 53,2 mg/kg) versus Carn alone at 17,6 mg/kg or 13,2 mg/kg. An important statistical difference was also observed between Carn at 17,6 mg/kg and Fidhycarn at 88,5 mg/kg groups.

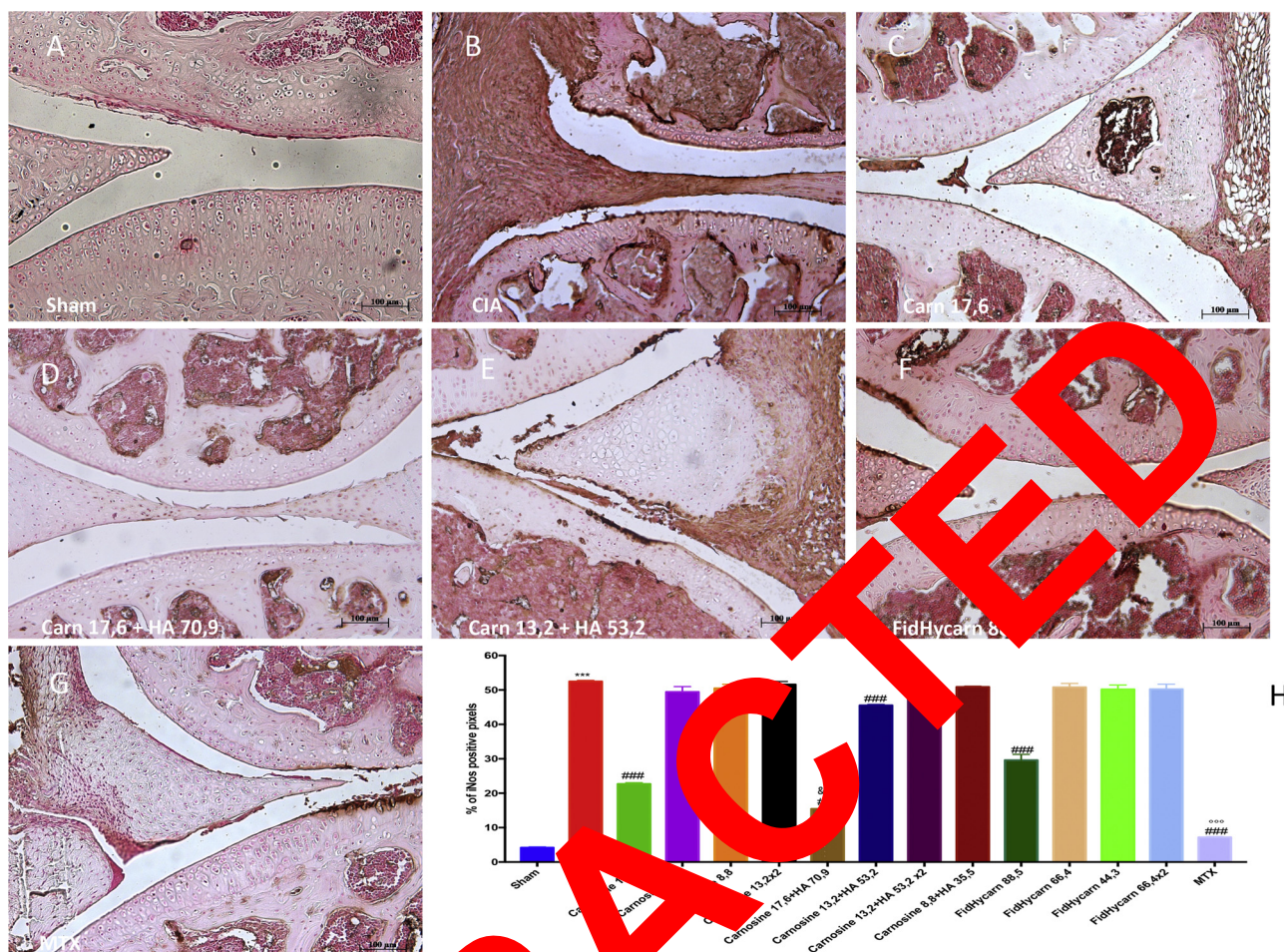


Fig. 10. The effects of Carn + HA association and FidHycarn on iNOS expression. Immunohistochemistry for iNOS in joint tissues respectively A sham group, B CIA + vehicle group, C, D, E CIA + Carn + HA association, F CIA + FidHycarn treatment group, G CIA + methotrexate. The results are expressed as % of positive pixels H. Figures are representative of at least three independent experiments. Values are means $\pm$ SEM of 20 animals for each group; ***P < 0,001 vs sham, #P < 0,05 vs CIA, ###P < 0,001 vs CIA. ****P < 0,0001 vs Carnosine 88,5 mg/kg versus methotrexate, &&&p < 0,001 versus Carnosine 17,6 mg/kg, \$\$\$p < 0,001 versus Carnosine 13,2 mg/kg.

3.7. Effects of Carn, Carn + HA association and FidHycarn on nitrotyrosine, PAR, iNOS and COX-2 expression

Significant increase in expression of nitrotyrosine, PAR, iNOS and COX-2 were found in CIA vehicle mice (Figs. 8–11B and see H). The treatment with Carnosine at lower doses of 13,2 also double administration and 8,8 mg/kg were able to reduce these expressions data not shown (see graphs 8,9,10,11 H), while reduced expressions of nitrotyrosine, PAR, iNOS and COX-2 were observed in CIA mice treated daily with Carnosine 17,6 mg/kg (Figs. 8–11C and see H). In addition, Carn + HA (17,6 + 70,9 mg/kg and 13,2 + 53,2 mg/kg) association was able to markedly reduce positive immunostainings for nitrotyrosine, PAR, iNOS and COX-2 (Figs. 8–11D, E and see H) where as Carn + HA association at lower doses of 8,8 + 35,5 mg/kg was not data not shown, see graphs 8,9,10,11 H. The oral treatment of FidHycarn at higher dose of 88,5 mg/kg more significantly reduced the positive immunostainings from CIA mice respect to lower doses 66,4 and 44,3 mg/kg (Figs. 8–11F and see H). Anyway, there was a statistical difference between FidHycarn and MTX (positive control) (Figs. 8–11G and see H).

4. Discussion

RA is an inflammatory disease with chronic inflammation of the synovial joints, proliferation of synovial cells and infiltration of

activated immunoinflammatory cells that lead to progressive devastation of cartilage and bone [43]. Existing treatments include NSAIDs, DMARDs and biologics, but none are beneficial and there is a significant ‘non-response’ rate [44]. Many studies have demonstrated a role of ROS in the pathogenesis of inflammatory chronic arthropathies, such as RA [45]. Antioxidant therapy or therapeutic coadministration of antioxidants along with conventional drugs may also represent therapeutic modalities in future management of RA patients [46]. Thus, new ways of supplementary or combinatory therapy of RA are of great meaning.

HA is extensively distributed in particular in extracellular matrix and body fluids. It is involved in the viscoelasticity of the fluid and elasticity in connective tissues, for example in cartilage and between cartilage surfaces [47]. In addition, in regular diarthrodial joints, HA represents not only a mechanical/physical barrier, but anti-inflammatory and analgesic effects have been demonstrated mitigating prostaglandin- or bradykinin-induced pain in experimental OA animals [47]. On the other hand, the degradation of HA could have a pathophysiological role in inflamed joints [13,48].

Based on our results, we demonstrated the synergistic effects of a new molecule, obtained by covalent conjugation of carnosine with hyaluronic acid (FidHycarn) with biopharmacological effects in an in vivo CIA model that are comparable to the methotrexate treatment, at the clinical dose per kg, for the majority of the evaluated parameters with no statistical significant difference. The inhibitory activity of HA against the action of serum carnosinases allows for a remarkable

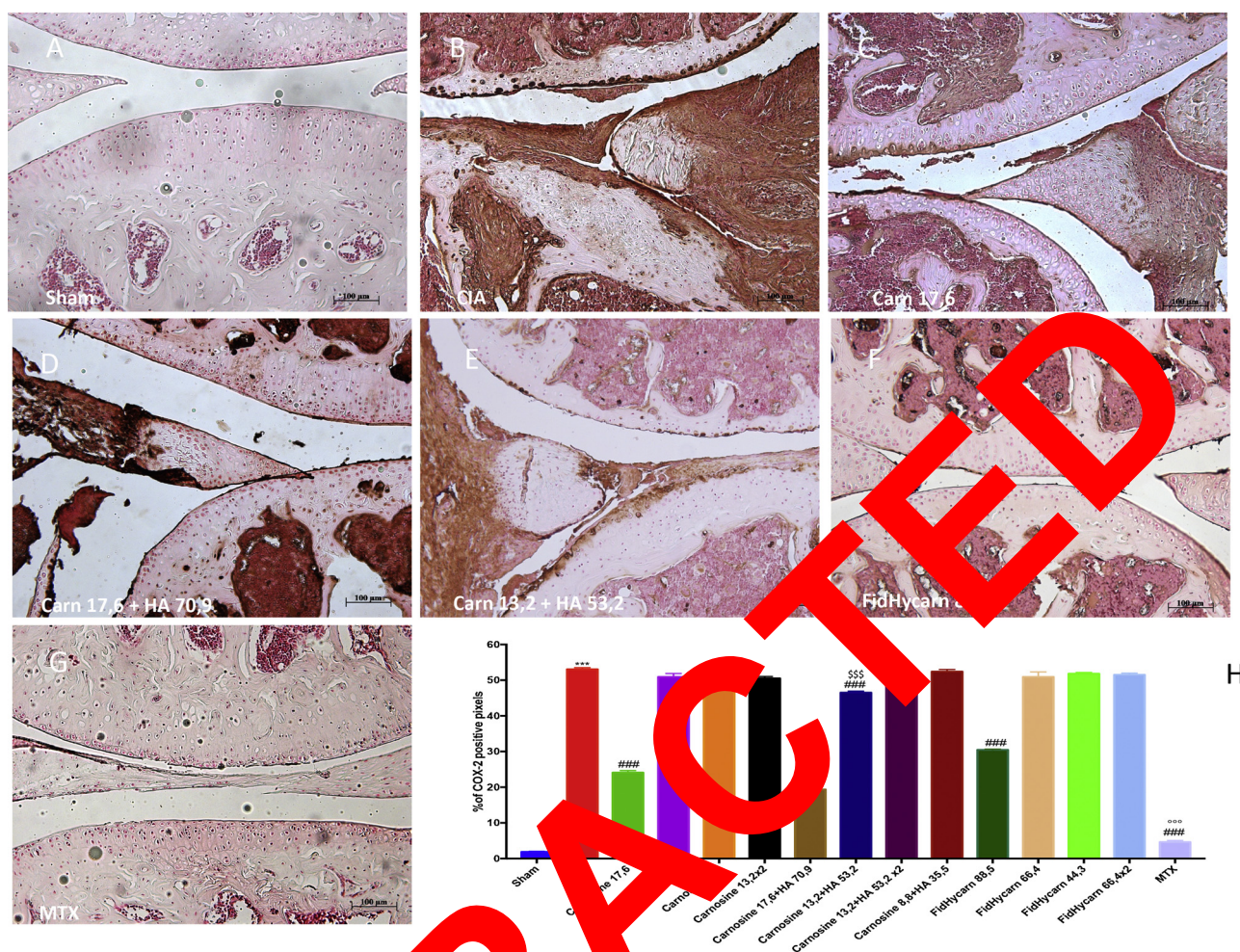


Fig. 11. The effects of Carn + HA association and Folicarn on COX-2 expression. Immunohistochemistry for COX-2 in joint tissues respectively (A) sham group, (B) CIA + vehicle group, (C, D, E) CIA + Carn + HA association, (F) Folicarn treatment group, (G) CIA + methotrexate. The results are expressed as % of positive pixels (H). Figures are representative of at least three independent experiments. Values are means $\pm$ SEM of 20 animals for each group; ***P < 0.001 vs sham, #P < 0,05 vs CIA, ###P < 0,001 vs CIA, ****p < 0,0001 vs CIA, ****p < 0,0001 vs Folicarn 88,5 mg/kg versus methotrexate, &&&p < 0,001 versus Carn 17,6 mg/kg, \$\$\$p < 0,001 versus Carn 13,2 mg/kg.

stability and consequently a higher activity in the unconjugated carnosine [49]. In that regard, here, we demonstrated that the oral administration of association of Carn + HA and of FidHycarn was able to ameliorate behavioral deficits as well as reduce macroscopic, histological and radiographical alterations in a more significant way compared to the single administration of either carnosine showing a possible synergistic action. The synergistic effect of FidHycarn on radiographic damage as well as significant improvement on behavioral deficit were just evidenced in the last CIA, a result that is not reached for the treatment of Carn + HA.

Oxidative stress has a key role in inflammation and destruction of arthritic animal joints and RA patients [50]. In this regard, high levels of ROS and protein oxidation markers and lipids are present in arthritic animals. The presence of ROS was detected in the serum of RA patients and also in the brain, liver and vascular tissues of rats with experimental arthritis [50]. There are numerous sources of ROS in inflammation; various inflammatory cytokines such as TNF- α , IL-1 and IL-6 can trigger NADPH oxidase to make known, occasionally toxic, quantities of ROS (O_2^-) which transmit their signals that stimulate transcription factors. It is known that the proinflammatory cytokines (IL-1 β , IL-6, and TNF- α), as well as the chemokines (MIP-1 α and MIP-2), are stated at spots of inflamed joints and determine the progression of chronic joint inflammation. It has been demonstrated that MCP-1, MIP-1 α , and MIP-1 β are chemotactic for lymphocyte groups [2] and are

expressed in tissue of patients with RA [51]. Interestingly, the association of Carn + HA and of FidHycarn demonstrated a more definite inhibition of proinflammatory cytokines and chemokines levels and a reduction of leukocyte penetration (MPO activity assay) compared to carnosine alone. The function of ROS in degradation of cartilage and bone is well-known [52]. Several evidences support a link among chondrocyte lipid peroxidation and cartilage oxidation/degradation [53]. ROS can instigate DNA damage which provoke poly ADP ribose synthase activation and cell loss [54]. Carn has showed inhibitory action on the oxidation of human LDL induced by Cu (II) and scavenger activity [49]. However, regarding the mechanism which allows carn to counteract the pathological effects of oxidative and nitrosative stress, a direct interaction with nitric oxide could be one of the probable paths. In addition, the effectiveness of carn in lessening the activation of PARP-1 or 2 after oxidative stress induction has been described in various studies [55]. In that regard, a previous work demonstrated that D- carnosine treatment was able to reduce nitrotyrosine and iNOS expression in a model of spinal cord injury [21]. Here, we observed a severe immunostaining of nitrotyrosine and a substantial lipid peroxidation with a structural adjustment of joint, maybe because of the formation of exceedingly reactive nitrogen byproducts. These results were similar to that reported in other our previous studies [31,56]. The association of Carn + HA and of FidHycarn was able to reduce in a more significant way nitrotyrosine formation, iNOS, PAR expression and

MDA levels. In the chronic arthritis such as RA or OA, high levels of cyclooxygenase (COX)-2 are detected [57]. COX-2, is severely up-regulated by inflammation and is involved in the producing of prostanoids (PGs), which are mediators of numerous characteristic features of inflammation and reactions leading to the tissue injury [58]. In the arthritis, the presence of COX-2 in infiltrating mononuclear inflammatory cells, in vascular endothelial cells and subsynovial fibroblast-like cells are well observed [59]. Some researchers have reported that (IL)-1 and (TNF)- α could regulate COX-2 expression [60]. In that regard, increased expression of COX-2 was found in rats subjected to CIA while the oral treatment of Carn + HA and of FidHycarn was able to markedly reduced COX-2 expression compared to carnosine alone.

5. Conclusions

In conclusion, we demonstrated that oral treatments of Carnosine + HA association and of FidHycarn at higher dose of 88,5 mg/kg were able to reduce the joint inflammation during CIA in a more significant way compared to all others. The effects of FidHycarn were only in some cases statistical different to Methotrexate used as positive control. In that regard, the conjugation of Carnosine with HA through its synergistic action, could represent a new therapeutic strategy to control the inflammatory conditions associated to autoimmune disorders such as arthritis. Thus, this study demonstrated for the first time that the HA-Carn conjugate, FidHycarn, constituted by two natural antioxidants and safe products: Carn and HA, had relevant beneficial effects in CIA animal model, similar to MTX, a standard treatment for RA but with proven side effects [10].

Availability of data and material

The datasets generated and/or analyzed for the present study are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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