Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at *Appl. Sci.* **2020**, *10*, 1324; doi:10.3390/app10041324

Article

Protective effect of new formulation of Carnosine+Hyaluronic acid on the inflammation and cartilage degradation in the experimental model of osteoarthritis.

Rosalba Siracusa[1], Daniela Impellizzeri[1], Marika Cordaro[1], Alessio F. Peritore [1], Enrico Gugliandolo[1], Ramona D'amico[1], Roberta Fusco[1], Rosalia Crupi[1], Enrico Rizzarelli[2], Salvatore Cuzzocrea[1,3*] Susanna Vaccaro[4], Mariafiorenza Pulicetta[4], Valentina Greco[2], Sebastiano Sciuto[2], Antonella Schiavinato[5], Luciano Messina[4] and Rosanna Di Paola[1]

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Science, University of Messina, Messina, Italy
[2] Department of Chemical Sciences, University of Catania, Viale A. Doria 6, 95125 Catania, Italy
[3] Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine,Saint Louis, USA
[4] Fidia Farmaceutici Contrada Pizzuta Noto (SR) Italy
[5] Fidia Farmaceutici Via Ponte della Fabbrica Abano Terme (PD) Italy
*Correspondence: salvator@unime.it; Tel.: +39 090-6765208

Abstract: Osteoarthritis (OA) is a disease that currently has no cure. There are numerous studies showing that carnosine and hyaluronic acid (HA) have a positive pharmacological action during joint inflammation. For this reason, the goal of this research was to discover the protective effect of a new HA+Carnosine formulation (FidHycarn) on the inflammatory response and on the cartilage degradation in *in vivo* experimental model of OA. This model was induced by a single intra-articular (i.ar.) injection of 25µl normal saline having 1mg of monosodium iodoacetate solution (MIA) in the knee joint. MIA injection caused histological alterations and degradation of cartilage as well as behavioral changes. Oral treatment with FidHycarn ameliorated the macroscopic signs, improved thermal hyperalgesia and weight distribution of hind paw as well as decreased histological and radiographic alterations. The oxidative damage was analyzed by evaluating the levels of nitrotyrosine and inducible nitric oxide synthase (iNOS) that were significantly reduced in FidHycarn rats. Moreover, the levels of pro-inflammatory cytokines and chemokines were also significantly reduced by FidHycarn. However, interestingly, in more cases, the effects of FidHycarn were not statistically different to Naproxen used as positive control. Thus, the new formulation containing Carnosine and HA could represent an interesting therapeutic strategy to combat osteoarthritis.

Keywords: osteoarthritis; carnosine; hyaluronic acid; inflammation; oxidative stress

Introduction

Osteoarthritis (OA) is one of the most common of arthropathies and the leading cause of disability with a large socioeconomic cost. OA is a condition which can affect any joint in the body. OA generally affects the joints that support most of the weight (such as knees and feet) and the joints we use the most (e.g. hand joints). In a healthful joint, the cartilage shelters the surface of the bones and supports the bones to move liberally against each other. When a joint develops OA, part of the cartilage tapers and the surface becomes coarser. This means the articulation doesn't move as easily as it should. When cartilage becomes worn out or damaged, all the tissues within the articulation

© 2020 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

become more active than usual as the body attempts to repair the damage. However, the reparation processes don't always operate so well and modifications to the joint structure can occasionally cause or contribute to symptoms such as swelling, pain and, ultimately, disability. One of the leading characteristics of OA is the collagen deterioration , as suggested by augmented tissue swelling and loss of proteoglycans [1]. Both collagen destruction and loss of proteoglycans unfavorably affect the mechanical properties of cartilage. Chondrocytes respond to tissue injury by increasing collagen and proteoglycan synthesis in an attempt to repair it [2]. If repair fails, damage will progress to articular cartilage degeneration. It's still not clear exactly what causes osteoarthritis. We do know it isn't just 'wear and tear' and that your danger of developing OA depends on an amount of factors. Furthermore, these degradation processes are thought to be largely elicited through excess production of pro-inflammatory and catabolic mediators. Among them, interleukin-1β (IL-1β) has been demonstrated to be predominantly involved in disease initiation and progression [3,4]. Oxidative stress shows a fundamental role in supporting cartilage degradation. In chondrocytes of the joint, data implicates reactive oxygen species (ROS) as signaling intermediates of IL-1β [5]. It has been proposed that ROS produced internal the joints may contribute notably to the pathogenesis of OA, since these inorganic oxidants are able to deteriorate cartilage through oxidation of extra-cellular matrix (ECM) components or post-translational modification (PTM) of metalloproteinases (MMPs) [6,7]. The existing treatments for postponing OA and RA progression comprise some disease modifying anti-rheumatic drugs (DMARDs) and biological agents that operate as immunomodulatory drugs in RA [8], several also act by inhibiting endothelial cell proliferation and cytokines [9]. Moreover, all of these compounds have potentially grave side effects and there are substantial differences in toxicity among DMARDs [10]. Hyaluronan (HA) is a foremost component of synovial fluid (SF) necessary for proper function of joints. Yet, owing to the fact that SF does not have any hyaluronidases, it is considered that ROS could be involved also in HA catabolism in the inflamed articulation. Whatever transition metal, e.g. copper or iron, can show an active function in oxidative HA catabolism. The polymer functions of HA are dimension specific and its fragments represent an information-rich system. The inter-mediate-sized HA-polymer fragments are inflammatory, immune-stimulatory and highly angiogenic [11]. Consequently, substances preventing HA from being degraded could have anti-inflammatory and anti-angiogenic properties. Carnosine (CARN), a fundamental endogenous molecule, has several physiological functions: pH buffering, radical scavenging, anti-glycating, heavy metal chelating and neutralization of toxic aldehydes. CARN will be found to have neuroprotective, cataract treating, hepatoprotective and anti-aging abilities [12], but its anti-inflammatory strength in auto-immune systemic inflammatory diseases, has been recently investigated. The ambition of this study is to explore the effect of carnosine-HA formulation on the inflammatory response and on the cartilage degradation in the experimental rat model of OA.

Material and Methods

Animals

Sprague–Dawley male rats (200–230 g, 7 weeks old, Envigo, Udine, Italy), were used for this study. Ten rats were used for each treatment group (see below). Food and water were available ad libitum. The University of Messina Review Board for the care and use of animals. Animal care was in accordance with Italian regulations on safety of animals used and other scientific purposes (D.Lgs 2014/26 and EU Directive 2010/63).

Osteoarthritis induction and treatment

OA was induced by intra-articular (i.ar.) injection of MIA in the knee joint as previously described [13]. First day, rats were anesthetized and only one injection of 25µl germ-free normal saline containing 1mg MIA was injected into right knee articulation through infra-patellar ligament. The left knee received an identical volume of 0.9% saline. MIA and drug solutions were prepared

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

under germ-free conditions and injected with a 50µl Hamilton microliter syringe with a 6mm, 27-gauge needle that was introduced into the joint for circa 2 to 3mm.

Experimental groups:

Rats were distributed into the following experimental groups:

Sham - Control: rats were exposed to intra-articular injection with 0.9% saline (25µl) instead of MIA and were orally treated with vehicle (distilled water) every day starting from day 3 after MIA induction; (n=20)

MIA - Vehicle: rats were subjected to MIA (as indicated below) (1 mg/kg) and were orally treated with distilled water (vehicle for FIDHYCARN) every day starting from day 3 after MIA induction; (n = 20)

MIA - Carnosine: animals were subjected to MIA (1 mg/kg) and received orally Carnosine (17.6 mg/kg) every day starting from day 3 after MIA induction; (n = 20)

MIA - HA: rats were subjected to MIA (1 mg/kg) and received orally HA (70.9 mg/kg) every day starting from day 3 after MIA induction; (n = 20)

MIA - Carnosine+HA: animals were subjected to MIA (1 mg/kg) and received orally Carnosine+HA (17.6 + 70.9 mg/kg) every day starting from day 3 after MIA induction; (n = 20)

MIA – FidHycarn (HA+Carnosine formulation): rats were subjected to MIA (1 mg/kg) and will received orally FIDHYCARN (88.5 mg/kg) every day starting from day 3 after MIA induction; (n = 20)

MIA - Naproxen: rats were subjected to MIA (1 mg/kg) and treated orally with Naproxen (10 mg/kg) every day starting from day 3 after MIA induction. (n = 20)

Synthesis of FidHycarn

The synthesis of FidHycarn is described in the patent WO2019069258 [14]. Briefly, the first step is the synthesis of carnosine methyl ester. 1.5 gr of carnosine was treated under stirring inside a 250ml flask with 50ml 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. 20ml 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, 1.1g of about 700 kDa hyaluronic acid were introduced in a ractor with 80ml of a mixture of H_2O and DMSO. Successively, a solution of H_2O and DMSO containing tris 2-(2 methoxethoxy) ethyl amine, 3-hydroxy 1,2,3 benzotriazin 4 (3H) 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.

100mg of the FidHycarn batch used in this in vivo study are constituted by 19.89mg of carnosine and 80.11mg of hyaluronic acid.

Behavioral Analysis

Hyperalgesic reactions to heat were determined at different time points (days 0, 7, 10, 13, 16 and 21) using a Basile Plantar Test. Each rat was limited to staying in a plexiglass chamber and was allowed to habituate. A portable unit consisting of a high intensity projector lamp was placed to deliver a thermal stimulation directly to an hind paw from under the chamber. The retraction latency period of injected paws was definite with an electronic clock circuit and thermocouple. Results were expressed as paw-withdrawal latency (s) [15].

Evaluation of Pain-Related Behavior

Pain connected with OA was characterized by changes in weight distribution on each hind paw [16].. Animals were positioned into a plexiglass chamber with each hind paw on the distinct force

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

plate, and they were allowed to habituate. When still, the force used on the plate by each hind paw was annotated. A total of four readings was taken for each rat at each time point (days 0, 7, 10, 13, 16 and 21) and the mean was used for calculation. % weight distribution of left (ipsilateral) hind paw was calculated by the following formula:

% weight distribution of left hind paw = left weight **/** (left weight **+** right weight) **x** 100

Macroscopic examination of the MIA induced OA site

The rats were euthanized by giving excess anesthesia at the 21th day of post-MIA injection. The whole right knee joint of all the animals was explanted for macroscopic study. Soft tissues surrounding the right knee were removed to observe the clear features of the articular cartilage. Macroscopic investigation was performed to find the deformities of the joint capsules [17].

Radiographic analysis

Radiographic analysis was performed by X-Ray Bruker FX Pro instrument (Milan, Italy). Radiographs were assessed by an observer in a blinded mode and scored by means of a semi-quantitative grading scale as previously indicated [18]. Briefly, articular space was scored by a scale 0–3 as 0 = regular, 1 = mild, 2 = partial, and 3 = whole loss of joint space. Subchondral bone sclerosis was indicated as 0 = normal, 1 = mild, 2 = moderate, and 3 = severe. Osteophytes formation was recorded as 0 = normal, 1 = osteophytes on the proximal tibia, 2 = osteophytes on the femoral condyle, and 3 = osteophytes present on both tibial and femoral condyle.

Histological Investigation

The 21st day post MIA administration, rats were killed by anesthetic overdose and tissues were fixed by transcardiac perfusion with 4% paraformaldehyde solution. Tibiofemoral joints have been taken and post-fixed in neutral buffered formalin (having 4% formaldehyde), were decalcified in EDTA and were treated as previously described. Mid-coronal tissue sections (5μm) were stained for valuations. Slices were stained with Hematoxylin and Eosin (H&E), and studied using light microscopy (Dialux 22 Leitz; Leica Microsystems SpA, Milan, Italy). Histopathological examination of the cartilage was assessed by the modified score of Mankin et al. (score range 0 to 12, from normal to complete disorganization and hypocellularity) [19]. Cartilage degeneration was assessed by staining with toluidine blue and analyzed using the following criteria described by Janusz et al. 1 = mild into the surface region; 2 = slightly extended in the upper center; 3 = moderate in the median area; 4 = extended area deep; and 5 = severe degeneration [20].

Immunohistochemistry

Briefly, slides for immunohistochemistry (7 μm) were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was slaked with 0.3% hydrogen peroxide in 60% methanol for 30 min. The slides were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Sections were incubated overnight with: anti-nitrotyrosine antibody (1:500 in PBS, w/v); anti-iNOS antibody (1:500 in PBS, w/v); anti- IL-1β (1:500 in PBS, v/v); anti- interleukin (IL)-6 (1:500 in PBS, w/v). Sections were washed with PBS, and will be incubated with secondary antibody. Specific labelling was identified with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex. The counter stain was developed with diaminobenzidine (brown) and nuclear fast red (red background). To verify that the immune-reactions for the nitrotyrosine is specific, we incubated some slices with the primary antibody in the presence of excess nitrotyrosine (10 mM). Similarly, to verify the binding specificity for iNOS, IL-1β , IL-6, we incubated several slices with only the primary antibody (no secondary) or with only the secondary antibody (no primary). 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 spontaneously plots a histogram profile of the deconvoluted DAB image, and a corresponding scoring log is demonstrated [21]. The

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

histogram profile corresponds to the positive pixel intensity value obtained from the computer program [22].

Measurement of cytokines

Tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β levels were evaluated in the plasma from MIA and sham rats. The assay was carried out by using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy).

Measurement of chemokines

On the 21st day post MIA administration levels of chemokines MIP-1α and MIP-2 were estimated in the aqueous joint extracts. In brief, articular tissues were homogenized on ice in 3ml lysis buffer (PBS including 2mM PMSF, and 0.1mg/ml (final concentration), each of antipain, aprotinin, pepstatin A and leupeptin) by means of Polytron (Brinkinarm Instruments, Westbury, NY, USA). After homogenization, the tissues have been centrifuged at 2.000g for 10min. Supernatants have been decontaminated with a Millipore filter (0.2 μm) and have been conserved at -80°C if they are not analyzed immediately. The extracts generally contained 0.2 to 1.5 mg protein/ml, as quantified with a protein-assay kit (Pierce Chemical Co., Rockford, IL, USA). The levels of MIP-1α and MIP-2 have been quantified by means of a modification of a double-ligand method. In brief, 96-well microtiter plates have been treated with 50μl/well of rabbit anti-cytokine antibodies (1μg/ml in 0.08N NaOH, 0.26 H_3BO_4, and 0.6M NaCl pH 9.6) for 16 hours at 4°C, and then have been laved with wash buffer (containing PBS, 0.05% Tween-20, pH 7.5). Nonspecific binding sites on plates have been blocked with 2% BSA in PBS and have been incubated for 90min at 37°C. Plates have been cleaned 4 times with wash buffer, and diluted aqueous joint samples (50 μl) were added, followed by incubation for 1 hour at 37°C. After wash of plates, chromogen substrate has been added. The plates have been incubated at room temperature to the wanted extinction, after which the reaction has been stopped with 50μl/well of 3M H_2SO_4 solution. The plates have been then read at 490 nm in an ELISA reader. This ELISA method consistently had a sensitivity limit of about 30 pg/ml.

Materials

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

Data evaluation

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 *in vivo* studies n represents the number of animals studied. In the experiments concerning histology or immunohistochemistry, the figures shown are illustrative of at least three experiments performed on different experimental days on the 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.

Results

Effects of FidHycarn on thermal hyperalgesia and weight distribution of hind paw after OA induction

Increased thermal hyperalgesia measured at different time points, as evidenced by a significant reduction in hind paw withdrawal latency as well as an important weight distribution asymmetry were observed in all MIA injected rats compared to sham. Oral treatments of Carnosine at 17.6 mg/kg, Carnosine+HA (17.6+70.9 mg/kg), HA (70.9 mg/kg) were not able to ameliorate behavioral deficits. Instead, oral FidHycarn treatment at 88.5 mg/kg showed significant behavioral improvements

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

(Figure 1A and B). No significant difference was found for hind paw withdrawal latency in FidHycarn treatment group vs Naproxen treatment group, while the difference is significant for weight distribution of hind paw.

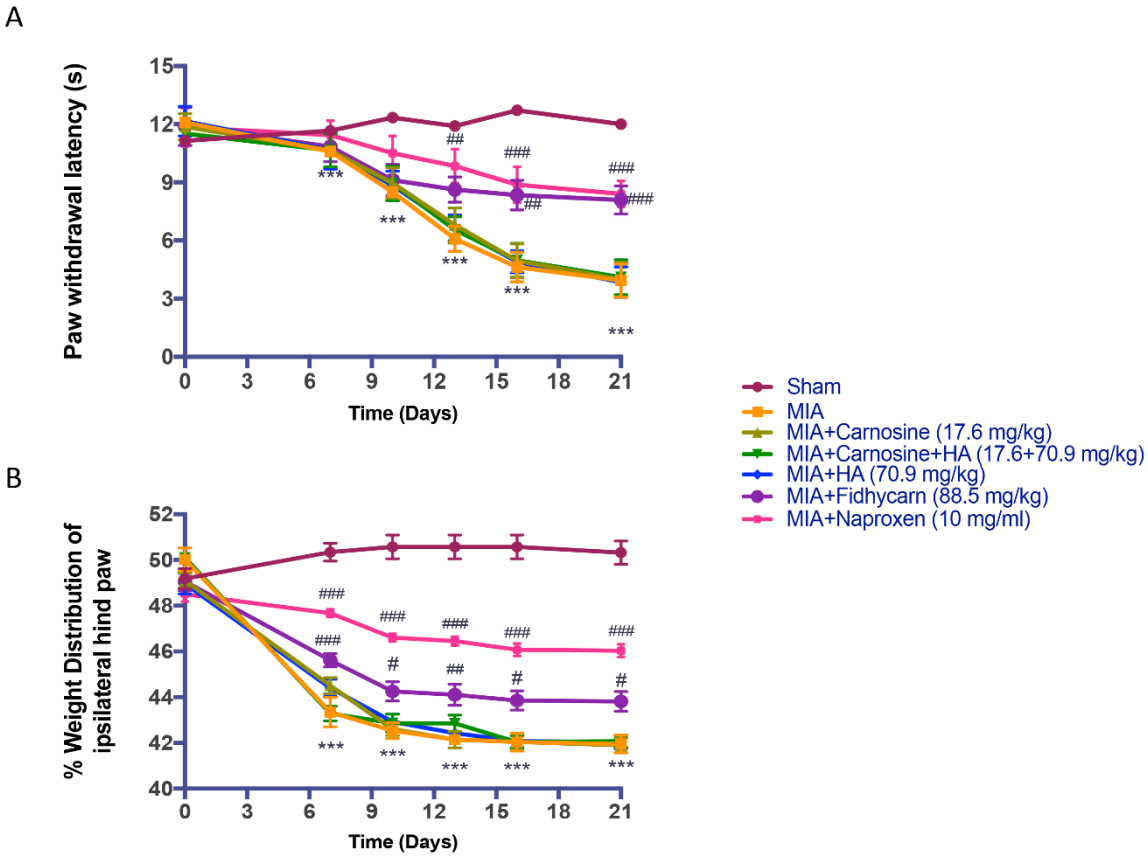

Figure 1. The effect of FidHycarn on behavior tests. Hyperalgesia was tested at the time-course indicated. Oral treatment with FidHycarn made a substantial improvement in hyperalgesia compared to other treatments (**A**). In addition, the treatment with FidHycarn showed a better distribution of the weight of the hind paw (**B**). ***p<0.001 *versus* Sham; ### p<0.001 *versus* MIA; ## p<0.01 *versus* MIA; # p<0.05 *versus* MIA.

Effects of FidHycarn on macroscopic and radiographic analysis after MIA injection

21 days after i.ar. injection of MIA, knee sections showed an important macroscopic and radiographic alteration in rats treated with vehicle. Oral treatments of Carnosine at 17.6 mg/kg, Carnosine+HA (17.6+70.9 mg/kg), HA (70.9 mg/kg) were not able to reduce these alterations. Instead, oral FidHycarn treatment at 88.5 mg/kg showed a substantial reduction of macroscopic and radiographic damage compared to the others. The effect of FidHycarn at 88.5 mg/kg was not statistical different compared to Naproxen treatment. No damage was discovered in Sham group (respectively Figure 2A and B and see radiographic analysis C).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

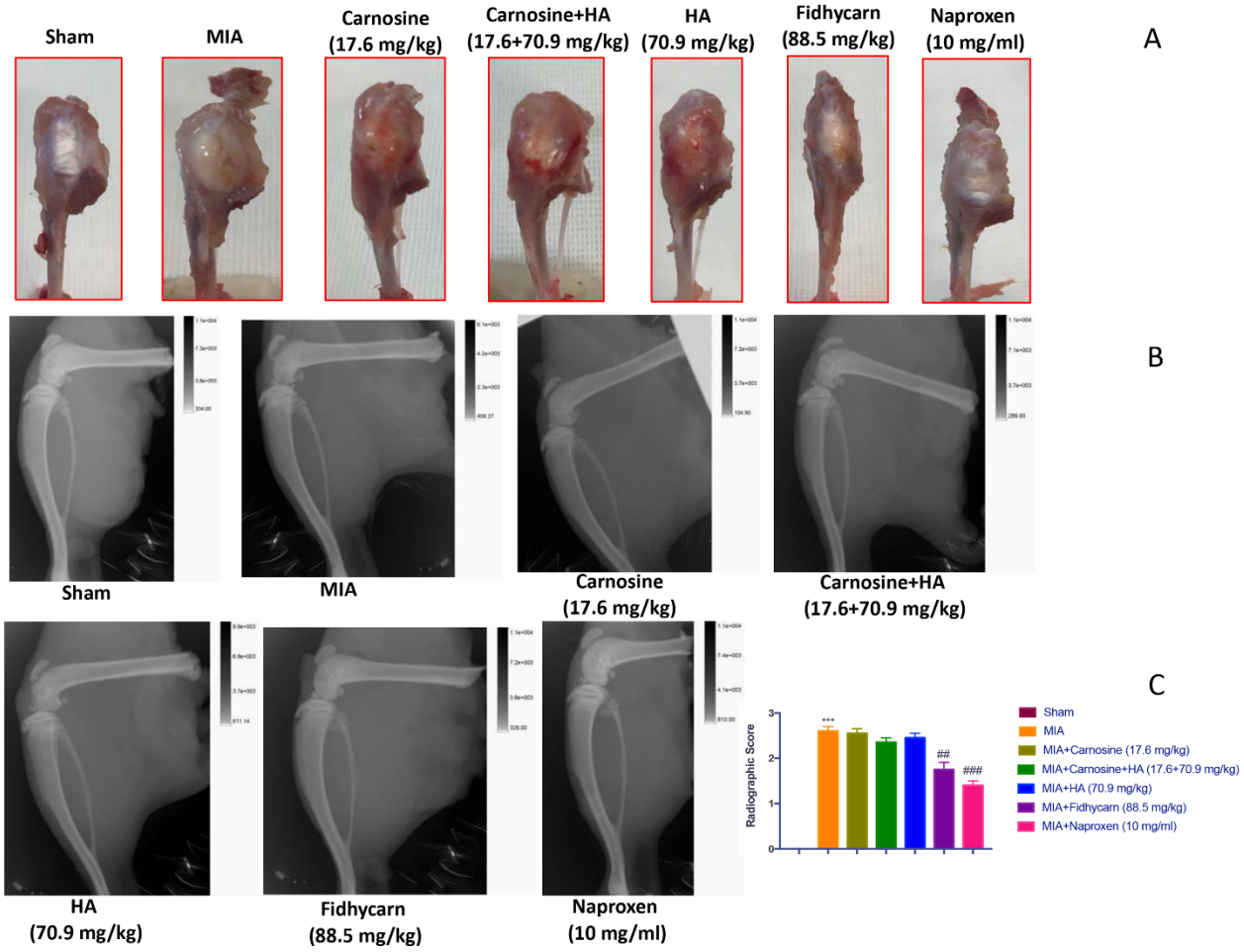

Figure 2. FidHycarn's macroscopic and radiographic effect on the knee joint. Macroscopic images for the thickening of joint capsule of the animals of different groups (**A**). Oral treatment with FidHycarn displayed a significant improvement of the articular cartilage. This improvement was also observed by radiographic investigation where a diminution in joint space was seen (**B**). Radiographic score (**C**). ***p<0.001 *versus* Sham; ## p<0.01 *versus* MIA; ### p<0.001 *versus* MIA.

Effects of FidHycarn on histological damage and cartilage degeneration after MIA injection

21 days after i.ar. injection of MIA, knee slices were stained with H&E. Histological investigation with light microscopy showed irregularities, disorganization, and fibrillation in the superficial layer and multilayering in transition and radial zones in the MIA injected rats in all experimental groups (Figure 3). In addition, cartilage degeneration was also evident by blue colour intensity of toluidine staining (Figure 4). Oral treatments of Carnosine at 17.6 mg/kg, Carnosine+HA (17.6+70.9 mg/kg), HA (70.9 mg/kg) were not able to reduce this histological alteration and cartilage degeneration. Instead, oral FidHycarn treatment at 88.5 mg/kg showed an essential reduction of histological damage and cartilage degeneration compared to the others. The effect of FidHycarn at 88.5 mg/kg on cartilage degeneration was statistically different compared to Naproxen treatment but no statistical difference was observed on histological damage. No injury was found in Sham group.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

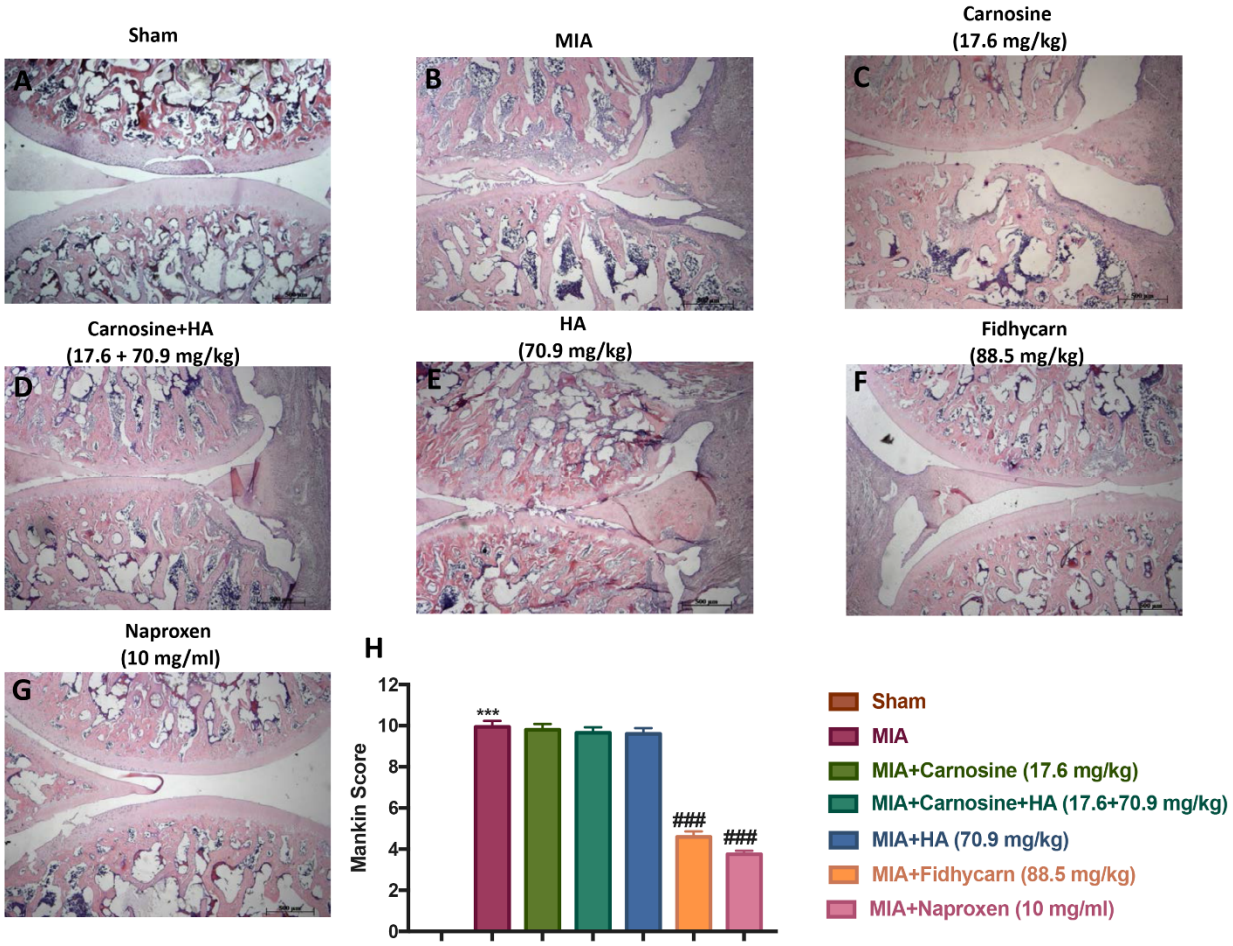

Figure 3. Effect of FidHycarn on histological features of OA knee tissue. Histological evaluation was performed by H&E staining. Panel (**A**), Sham; panel (**B**), MIA; panel (**C**), Carnosine; panel (**D**), Carnosine+HA; panel (**E**), HA; panel (**F**), FidHycarn; panel (**G**), Naproxen treatment. Figures are representative of all rats in each group. Panel (**H**), Mankin Score for the several treatment groups. ***p <0.001 *versus* Sham; ### p<0.001 *versus* MIA.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

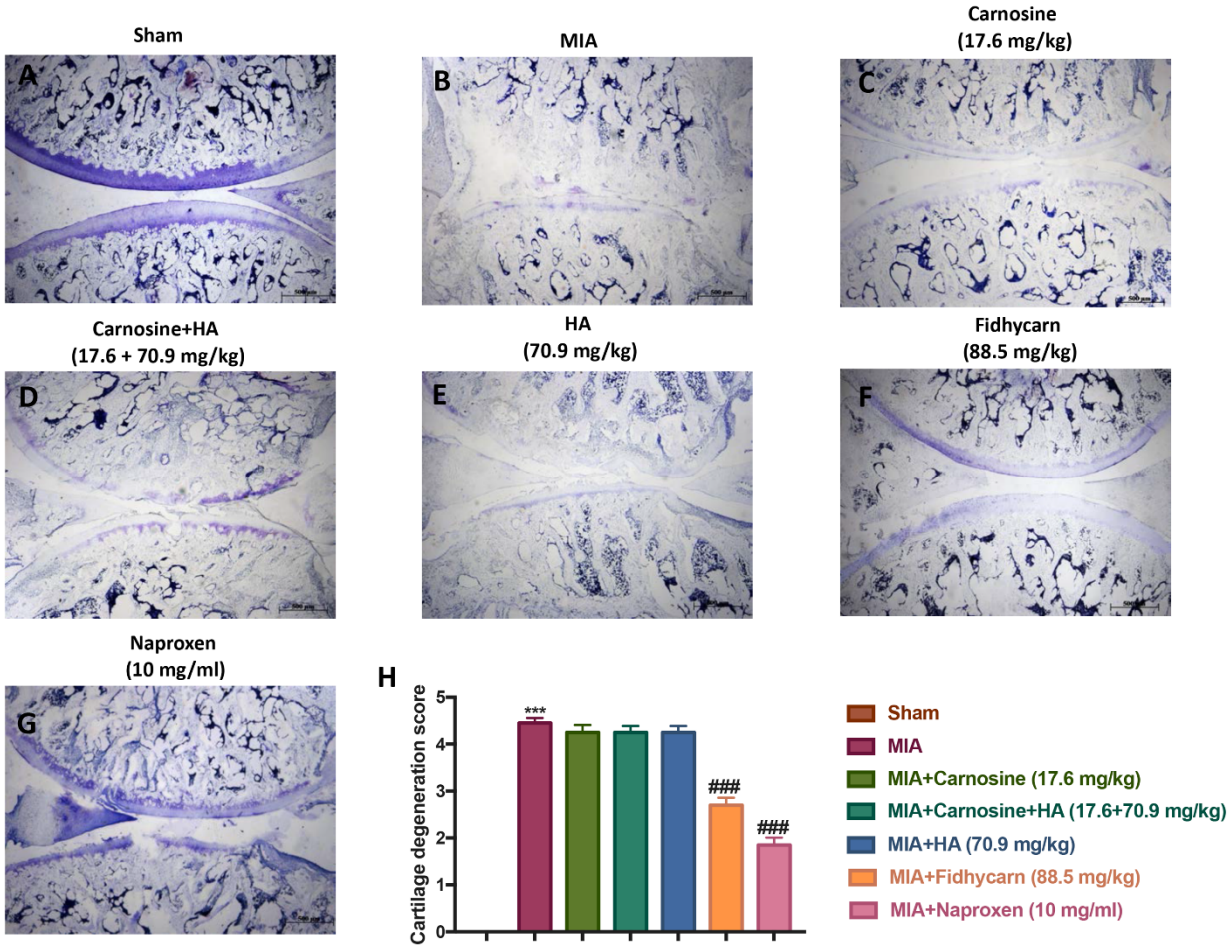

Figure 4. Effect of FidHycarn on cartilage degradation after MIA-induction. Cartilage evaluation by blue of toluidine-staining of joint sections showed significantly degradation in MIA mice (**B**) compared to Sham animals (**A**). Ameliorated cartilage alterations were observed in the sections from MIA-Carnosine, MIA-Carnosine+HA, MIA-HA (**C, D, E**) and more significantly in FidHycarn treated mice (**F**). Naproxen is a positive control (**G**). Cartilage degradation score is shown in **H**. Figures are representative of all animals in each group. ***p<0.001 *versus* Sham; ### p<0.001 *versus* MIA.

Effects of FidHycarn on cytokines and chemokines levels after OA induction

A substantial increase in TNF-α, IL-1β, IL-6, MIP-lα and MIP-2 production was found in OA subjected rats 21 days post MIA injection. Levels of citokines and chemokines were not significantly reduced in MIA rats treated orally with Carnosine 17.6 mg/kg, Carnosine+HA (17.6+70.9 mg/kg) and HA (70.9 mg/kg). On the contrary, oral FidHycarn treatment was able to reduce these levels. The effect of FidHycarn at 88.5 mg/kg on TNF-α, IL-6 and MIP-1α levels was statistically different to Naproxen treatment but no statistical difference was observed on IL-1β and MIP-2 levels. Low levels were found in Sham animals (Figure 5).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

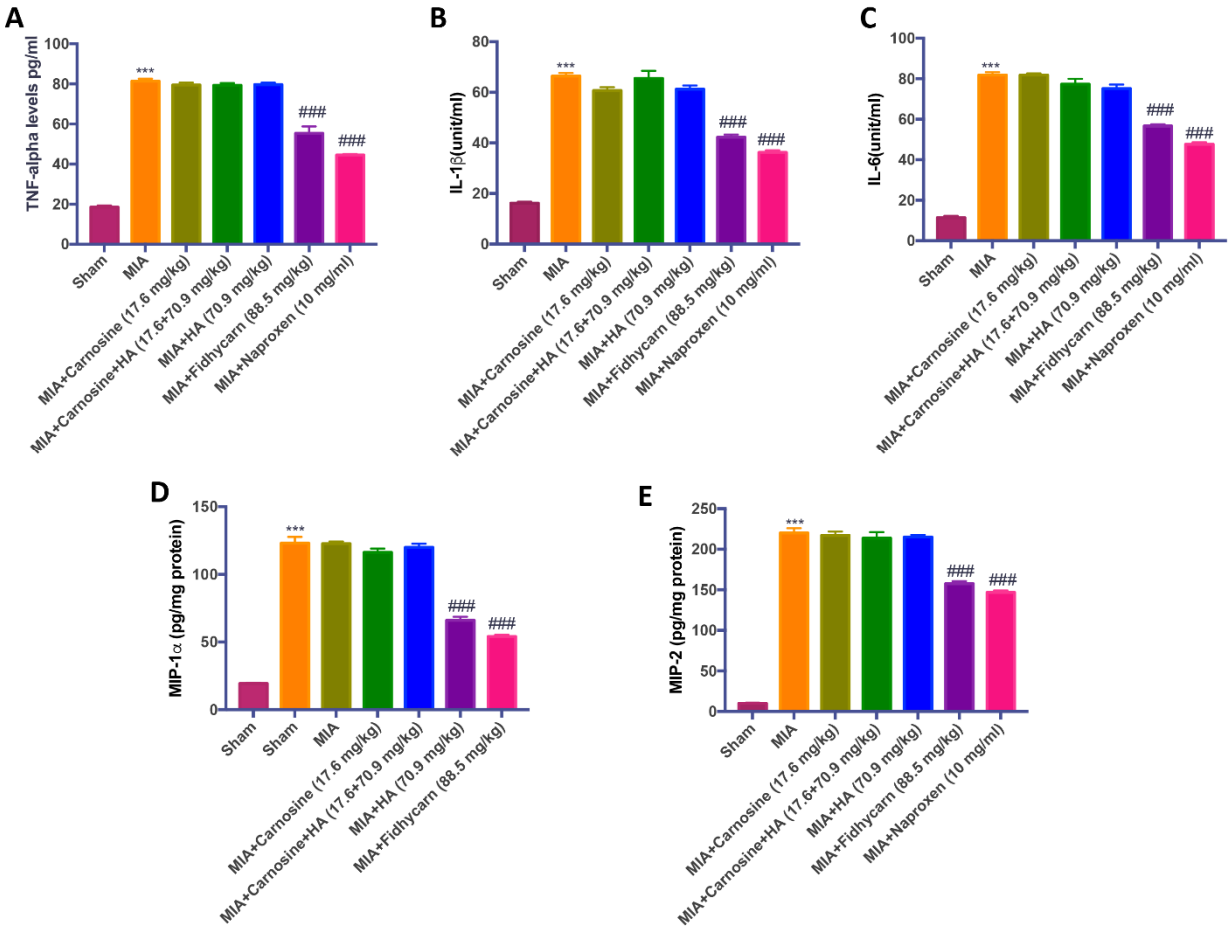

Figure 5. The effects of FidHycarn on cytokines and chemokines production. TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), MIP-lα (**D**) and MIP-2 (**E**) levels were shown. Values are given as mean ± SEM of 20 animals for each group ***p<0.001 *versus* Sham; ### p<0.001 *versus* MIA.

Effects of FidHycarn on nitrotyrosine, INOS, IL-1β and IL-6 expression after OA induction. A significant positive staining for nitrotyrosine, iNOS, IL-1β, IL-6 was found in MIA rats treated with vehicle (Figure 6, 7, 8, 9 B and see H). Oral treatments of Carnosine at 17.6 mg/kg, Carnosine+HA (17.6+70.9 mg/kg), HA (70.9 mg/kg) were not able to reduce these positive staining (Figure 6, 7, 8, 9 C, D, E and see H). Instead, oral FidHycarn treatment at 88.5 mg/kg showed an important reduction of positive staining for nitrotyrosine, iNOS, IL-1β and IL-6 compared to the others (Figure 6, 7, 8, 9 F and see H). The effect of FidHycarn at 88.5 mg/kg was statistically different to Naproxen treatment (Figure 6, 7, 8, 9 G and see H). No positive staining was discovered in Sham group (Figure 6, 7, 8, 9 and see H).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

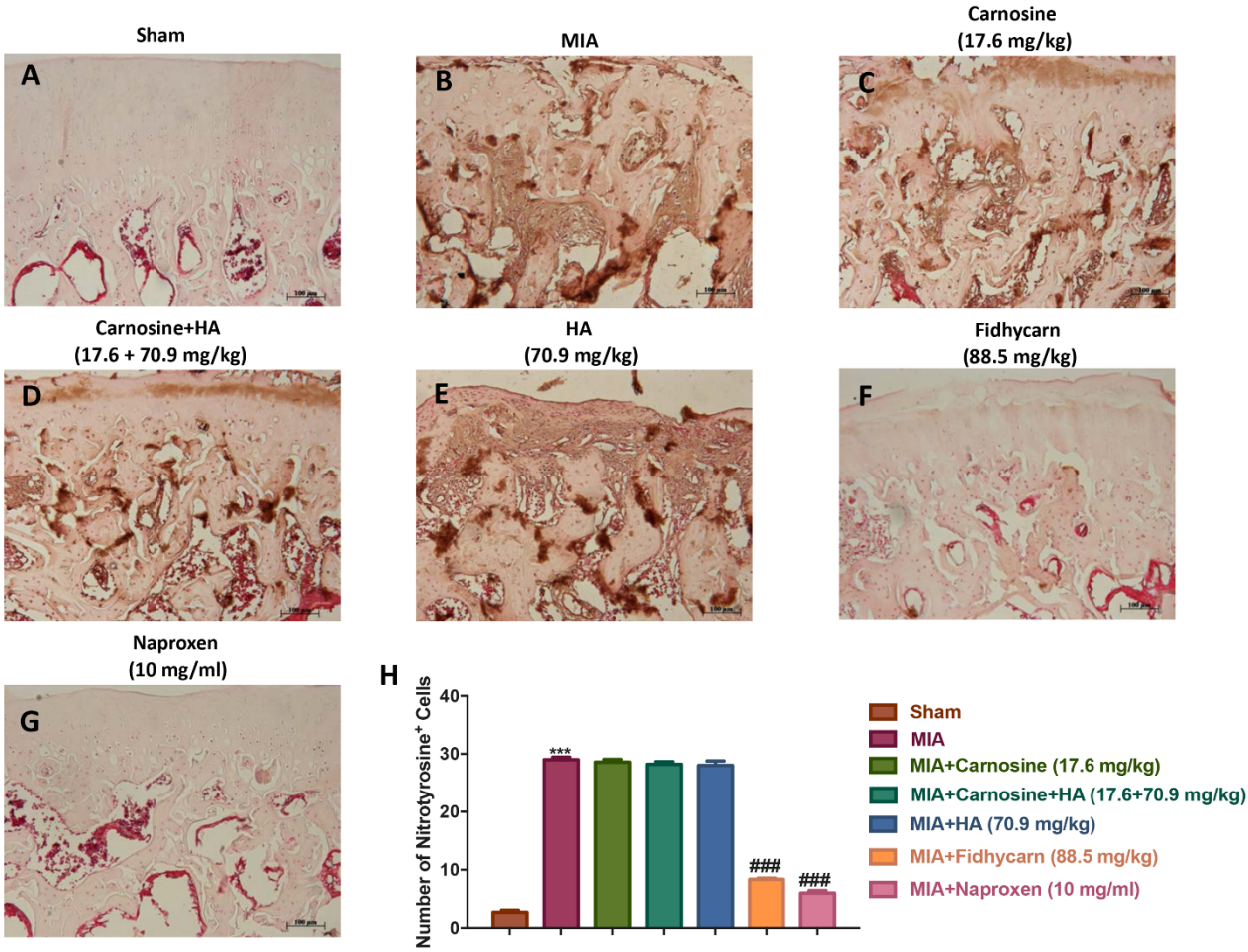

Figure 6. Effects of FidHycarn on nitrotyrosine expression. Immunohistochemistry for nitrotyrosine in joint tissues respectively (**A**) Sham group, (**B**) MIA group, (**C**) MIA+Carnosine, (**D**) MIA+Carnosine+HA, (**E**) MIA+HA, (**F**) MIA+FidHycarn treatment group, (**G**)MIA+Naproxen. The results are expressed as % of positive pixels (**H**). Figures are representative of at least three independent experiments. ***p<0.001 *versus* Sham; ### p<0.001 *versus* MIA.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

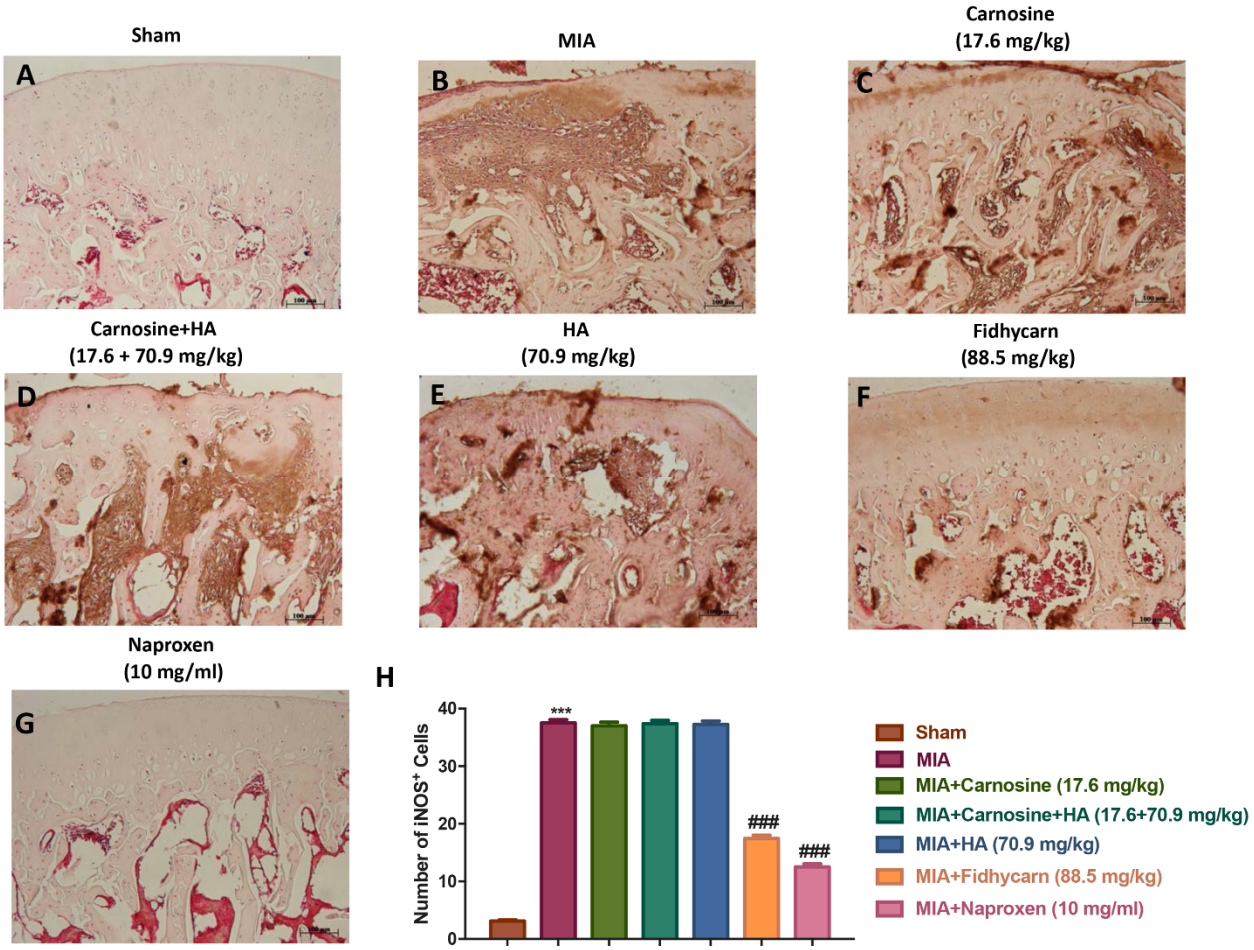

Figure 7. The effects of FidHycarn on iNOS expression. Immunohistochemistry for iNOS in joint tissues respectively (**A**) Sham group, (**B**) MIA group, (**C**) MIA+Carnosine, (**D**) MIA+Carnosine+HA, (**E**) MIA+HA, (**F**) MIA+FidHycarn treatment group, (**G**)MIA+Naproxen. The results are expressed as % of positive pixels (**H**). Figures are representative of at minimum three independent experiments. ***p<0.001 *versus* Sham; ### p<0.001 *versus* MIA.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

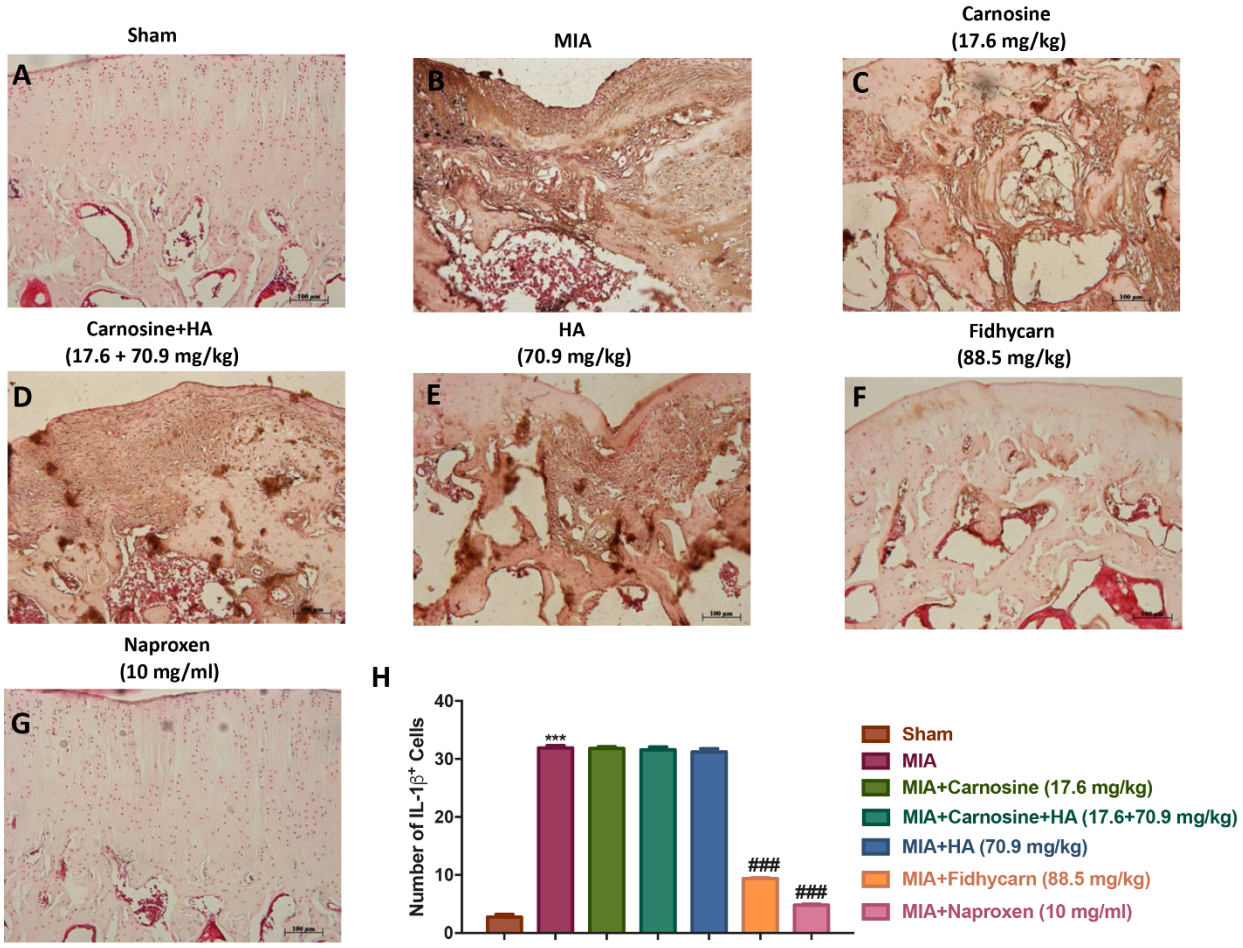

Figure 8. The effects of FidHycarn on IL-1β expression. Immunohistochemistry for IL-1β in joint tissues respectively (**A**) Sham group, (**B**) MIA group, (**C**) MIA+Carnosine, (**D**) MIA+Carnosine+HA, (**E**) MIA+HA, (**F**) MIA+FidHycarn treatment group, (**G**)MIA+Naproxen. The results are expressed as % of positive pixels (**H**). Figures are representative of at least three independent experiments. ***p<0.001 *versus* Sham; ### p<0.001 *versus* MIA.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

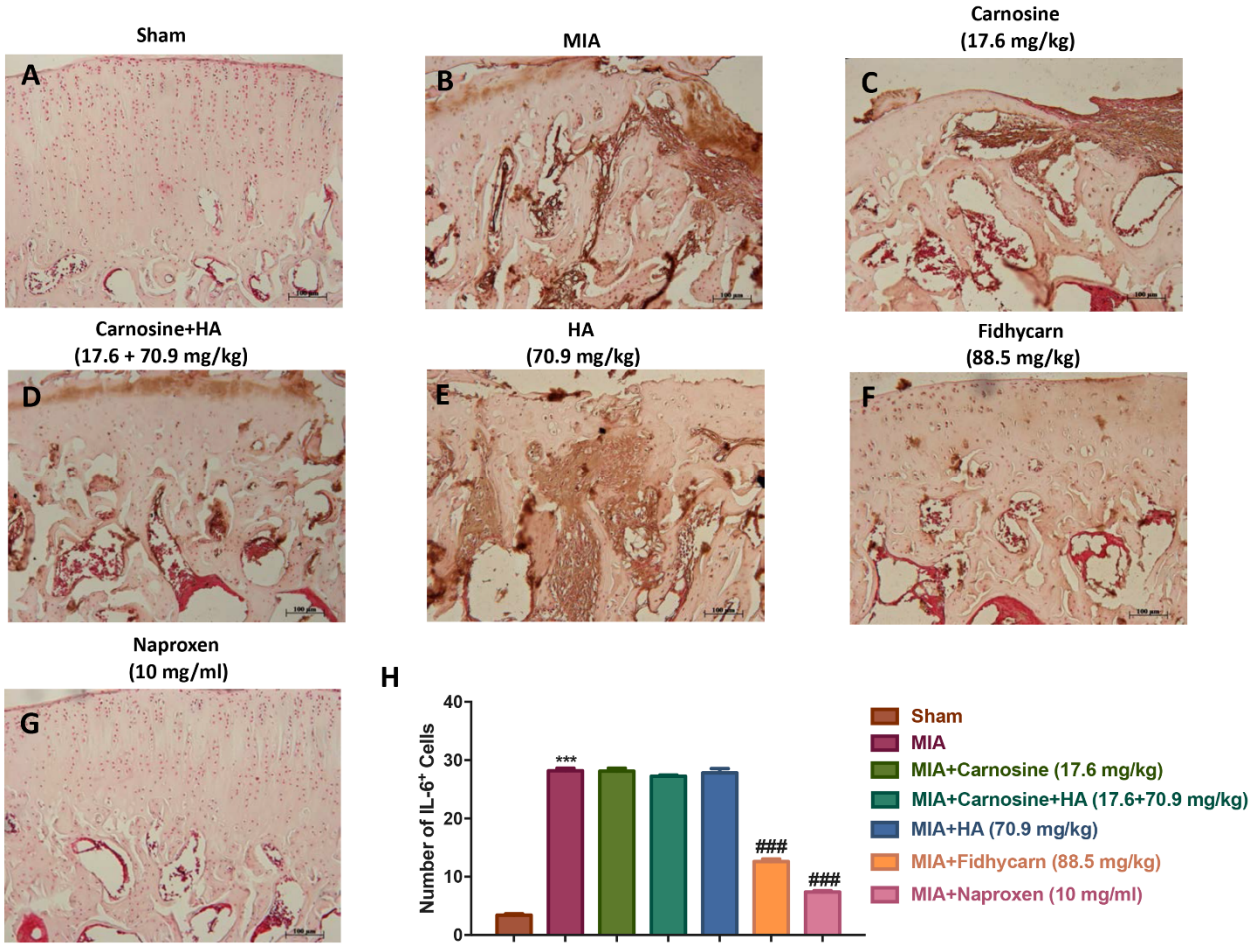

Figure 9. The effects of FidHycarn on IL-6 expression. Immunohistochemistry for IL-6 in joint tissues respectively (**A**) Sham group, (**B**) MIA group, (**C**) MIA+Carnosine, (**D**) MIA+Carnosine+HA, (**E**) MIA+HA, (**F**) MIA+FidHycarn treatment group, (**G**)MIA+Naproxen. The results are expressed as % of positive pixels (**H**). Figures are representative of at minimum three independent experiments. ***p<0.001 *versus* Sham; ### p<0.001 *versus* MIA.

Discussion

OA is a very common degenerative disease. This disease is characterized by structural loss and functional impairment of the joints leading to disability [23-25]. Whereas there is no cure for OA to date, most of the treatments available are intended at controlling pain and upholding joint function. Usually the treatment of OA begins with safer and less invasive therapies, such as physical activity. This is because the benefits of exercise can lead to prevention of obesity and maintenance of normal joint health [26,27]. If OA pain cannot be controlled by physical therapy, different pharmacological treatments are available. Paracetamol is usually the first line drug therapy in OA [28,29]. If paracetamol is not successful, the next level of pharmacological treatment varies according to the patient, but usually involves the use of non-steroidal topical or oral anti-inflammatory drugs (NSAIDs); topical capsaicin; injections of intra-articular corticosteroids and opioids [30,31]. However, these drugs expose patients to the risk of liver and kidney damage [29]. In addition, these medicines often offer only partial relief [32,33] and do not help the body rebuild damaged joint cartilage [34]. Numerous studies have shown a role of ROS in the pathogenesis of chronic inflammatory arthropathy, such as OA [35]. Therefore, researchers are considering a multifactorial approach to managing OA.

HA is secreted by chondrocytes and used for cartilage synthesis. According to the researchers, HA is helpful in the controlling of OA because it interferes with pain mediators and crucial enzymes

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

(such as metalloproteinase) responsible for the digestion and destruction of healthy cartilage tissue [36,37]. HA is generally administered intraarticularly; however, and also orally y [38-40]. New products are frequently being developed that modify the composition of the molecule and associate it with other drugs to maximize the effect [36,41]. CARN is a dipeptide consisting of β-alanine and L-histidine. The anti-inflammatory potential of CARN has been studied in autoimmune diseases [42]. Furthermore, the ability of CARN as antioxidant, anti-glycating and toxic metal-ion chelating properties is well documented [43]. In previous studies the capacity of CARN in the regression of the senescence of cultured human fibroblasts and in delaying aging has been highlighted, but the mechanisms remain uncertain [44]. Furthermore, it has been shown that CARN reduces oxidative damage on chondrocytes and prevents cartilage degradation in the "arthritic" joint [42]. However, the anti-inflammatory potential of CARN in OA is still poorly studied. In a recent report, the potential protective effect of an oral CARN supplement was assessed to improve Type 2 Diabetes-induced OA [45].

Based on the studies performed on HA and CARN, the aim of this study was to evaluate the synergistic effect of a new formulation called FidHycarn, consisting of the covalent conjugation of these two substances, in an *in vivo* MIA model. Furthermore, this study has demonstrated that FidHycarn has a bio-pharmacological effect comparable to the treatment with Naproxen, at the clinical dose per kg, for almost all the parameters assessed without significant statistical differences. In this regard we have shown that the oral administration of the conjugated formulation FidHycarn was able to improve behavioral deficits and to reduce macroscopic, histological and radiographic alterations in a more significant way compared to the administration of single compounds or Carnosine+HA association.

Oxidative stress shows a potential role on the pathogenesis of OA. In fact, in subjects suffering from OA there is a reduction in antioxidants [46]. Also proinflammatory cytokines (IL-1β, IL-6, and TNF-α), chemokines (MIP-Iα and MIP-2) and NO play an important function in the pathogenesis of joint damage [47-49]. In this regard, in this study, it was found that in rats with OAtreated with Carnosine or HA or Carnosine+HA the levels of pro-inflammatory cytokines and chemokines were not significantly reduced. In contrast, in FidHycarn-treated rats a significant diminution of these proteins was observed. The same trend was highlighted when the expression of nitrotyrosine and iNOS was evaluated.

In conclusion, this study has demonstrated that FidHycarn formulation at dose of 88.5 mg/kg was able to reduce the joint inflammation and the cartilage degeneration induced by MIA i.ar. in a more significant way compared to the of HA and/ or CARN treatments. The effects of FidHycarn were only in some cases statistical different to Naproxen used as positive control. Therefore we can say that probably the conjugation of HA with Carnosine increases its biological activity and protects the degradation of carnosine itself thanks to its antioxidant properties [50,51]. Then, the presence of HA in the FidHycarn conjugate in a synergistic way could enhance the action carnosine antioxidant, allowing a greater resistance to the action of serum carnosinases, guaranteeing a remarkable stability and consequently a higher activity than unconjugated carnosine. Thus, this study demonstrated for the first time that the FidHycarn formulation by using two safe products Carnosine and HA had important results similar to Naproxen, a standard treatment for OA but with proven side effects [52].

Acknowledgements: The authors would like to thank Miss Valentina Malvagni for editorial support with the manuscript.

Author contributions: Conceptualization, DP.R., S.A. and C.S.; Methodology, S.R., I.D., P.AF., G.E. F.R; Validation, C.S., S.R., I.D and DP.R.; Formal Analysis, S.R., C.M., R.E., V.S., P.M., G.V., S.S., M.L.; Investigation, S.R., I.D., C.M., DA. R.; Data Curation, DP.R., I.D., C.S., S.A.; Writing – Original Draft Preparation, S.R.; Writing – Review & Editing, I.D., S.A.; Visualization, C.R.; Supervision, DP.R., S.A., C.S.

Conflict of interest: The authors declare that there are no conflict of interest.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

References

1. Bank, R.A.; Soudry, M.; Maroudas, A.; Mizrahi, J.; TeKoppele, J.M. The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis and rheumatism* **2000**, *43*, 2202-2210, doi:10.1002/1529-0131(200010)43:10<2202::AID-ANR7>3.0.CO;2-E.

2. Nelson, F.; Dahlberg, L.; Laverty, S.; Reiner, A.; Pidoux, I.; Ionescu, M.; Fraser, G.L.; Brooks, E.; Tanzer, M.; Rosenberg, L.C., et al. Evidence for altered synthesis of type II collagen in patients with osteoarthritis. *The Journal of clinical investigation* **1998**, *102*, 2115-2125, doi:10.1172/JCI4853.

3. Goldring, M.B.; Marcu, K.B. Cartilage homeostasis in health and rheumatic diseases. *Arthritis research & therapy* **2009**, *11*, 224, doi:10.1186/ar2592.

4. Abramson, S.B.; Attur, M. Developments in the scientific understanding of osteoarthritis. *Arthritis research & therapy* **2009**, *11*, 227, doi:10.1186/ar2655.

5. Ahmad, R.; Sylvester, J.; Ahmad, M.; Zafarullah, M. Involvement of H-Ras and reactive oxygen species in proinflammatory cytokine-induced matrix metalloproteinase-13 expression in human articular chondrocytes. *Arch Biochem Biophys* **2011**, *507*, 350-355, doi:10.1016/j.abb.2010.12.032.

6. Kar, S.; Subbaram, S.; Carrico, P.M.; Melendez, J.A. Redox-control of matrix metalloproteinase-1: a critical link between free radicals, matrix remodeling and degenerative disease. *Respiratory physiology & neurobiology* **2010**, *174*, 299-306, doi:10.1016/j.resp.2010.08.019.

7. Tiku, M.L.; Shah, R.; Allison, G.T. Evidence linking chondrocyte lipid peroxidation to cartilage matrix protein degradation. Possible role in cartilage aging and the pathogenesis of osteoarthritis. *The Journal of biological chemistry* **2000**, *275*, 20069-20076, doi:10.1074/jbc.M907604199.

8. Seitz, M.; Loetscher, P.; Dewald, B.; Towbin, H.; Rordorf, C.; Gallati, H.; Baggiolini, M.; Gerber, N.J. Methotrexate action in rheumatoid arthritis: stimulation of cytokine inhibitor and inhibition of chemokine production by peripheral blood mononuclear cells. *Br J Rheumatol* **1995**, *34*, 602-609.

9. Koda, M.; Yoshino, S.; Nakamura, H.; Asano, G. [Effects of disease modifying antirheumatic drugs (DMARDs) and DEX on IL-1 beta and IL-6 production by IL-1 beta stimulated synovial culture cells]. *Nihon Ika Daigaku Zasshi* **1996**, *63*, 419-423.

10. Fries, J.F.; Williams, C.A.; Ramey, D.R.; Bloch, D.A. The relative toxicity of alternative therapies for rheumatoid arthritis: implications for the therapeutic progression. *Semin Arthritis Rheum* **1993**, *23*, 68-73.

11. Soltes, L.; Kogan, G. Catabolism of hyaluronan: involvement of transition metals. *Interdiscip Toxicol* **2009**, *2*, 229-238, doi:10.2478/v10102-009-0026-y.

12. Boldyrev, A.A. Protection of proteins from oxidative stress: a new illusion or a novel strategy? *Ann N Y Acad Sci* **2005**, *1057*, 193-205, doi:10.1196/annals.1356.013.

13. Vonsy, J.L.; Ghandehari, J.; Dickenson, A.H. Differential analgesic effects of morphine and gabapentin on behavioural measures of pain and disability in a model of osteoarthritis pain in rats. *European journal of pain* **2009**, *13*, 786-793, doi:10.1016/j.ejpain.2008.09.008.

14. SCHIAVINATO, A.I.; GRECO, V.I.; MESSINA, L.I.; VACCARO, S.I.; RIZZARELLI, E.I.; SCIUTO, S. Pharmaceutical composition containing hyaluronic acid and carnosine and relative use *Patent WO2019069258* **2019**.

15. Hargreaves, K.; Dubner, R.; Brown, F.; Flores, C.; Joris, J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **1988**, *32*, 77-88.

16. Izumi, M.; Ikeuchi, M.; Ji, Q.; Tani, T. Local ASIC3 modulates pain and disease progression in a rat model of osteoarthritis. *J Biomed Sci* **2012**, *19*, 77, doi:10.1186/1423-0127-19-77.

17. Kuroda, R.; Usas, A.; Kubo, S.; Corsi, K.; Peng, H.R.; Rose, T.; Cummins, J.; Fu, F.H.; Huard, J. Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells. *Arthritis and rheumatism* **2006**, *54*, 433-442, doi:10.1002/art.21632.

18. Ahmed, A.S.; Li, J.; Erlandsson-Harris, H.; Stark, A.; Bakalkin, G.; Ahmed, M. Suppression of pain and joint destruction by inhibition of the proteasome system in experimental osteoarthritis. *Pain* **2012**, *153*, 18-26, doi:10.1016/j.pain.2011.08.001.

19. Xu, Y.; Liu, Q.; Liu, Z.L.; Lim, L.; Chen, W.H.; Lin, N. Treatment with SiMiaoFang, an anti-arthritis chinese herbal formula, inhibits cartilage matrix degradation in osteoarthritis rat model. *Rejuvenation Res* **2013**, *16*, 364-376, doi:10.1089/rej.2013.1439.

20. Di Paola, R.; Fusco, R.; Impellizzeri, D.; Cordaro, M.; Britti, D.; Morittu, V.M.; Evangelista, M.; Cuzzocrea, S. Adelmidrol, in combination with hyaluronic acid, displays increased anti-inflammatory and analgesic effects against monosodium iodoacetate-induced osteoarthritis in rats. *Arthritis research & therapy* **2016**, *18*, 291, doi:10.1186/s13075-016-1189-5.

21. Sawant, S.; Gokulan, R.; Dongre, H.; Vaidya, M.; Chaukar, D.; Prabhash, K.; Ingle, A.; Joshi, S.; Dange, P.; Joshi, S., et al. Prognostic role of Oct4, CD44 and c-Myc in radio-chemo-resistant oral cancer patients and their

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

tumourigenic potential in immunodeficient mice. *Clin Oral Investig* **2016**, *20*, 43-56, doi:10.1007/s00784-015-1476-6.

22. Varghese, F.; Bukhari, A.B.; Malhotra, R.; De, A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PloS one* **2014**, *9*, e96801, doi:10.1371/journal.pone.0096801.

23. Seed, S.M.; Dunican, K.C.; Lynch, A.M.; Desilets, A.R. An update in options for the treatment of pain: a review of new opioid formulations. *Hosp Pract (1995)* **2012**, *40*, 166-175, doi:10.3810/hp.2012.02.957.

24. Lawrence, R.C.; Felson, D.T.; Helmick, C.G.; Arnold, L.M.; Choi, H.; Deyo, R.A.; Gabriel, S.; Hirsch, R.; Hochberg, M.C.; Hunder, G.G., et al. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. *Arthritis and rheumatism* **2008**, *58*, 26-35, doi:10.1002/art.23176.

25. Lane, N.E.; Brandt, K.; Hawker, G.; Peeva, E.; Schreyer, E.; Tsuji, W.; Hochberg, M.C. OARSI-FDA initiative: defining the disease state of osteoarthritis. *Osteoarthritis Cartilage* **2011**, *19*, 478-482, doi:10.1016/j.joca.2010.09.013.

26. Sinusas, K. Osteoarthritis: diagnosis and treatment. *Am Fam Physician* **2012**, *85*, 49-56.

27. Egan, B.A.; Mentes, J.C. Benefits of physical activity for knee osteoarthritis: a brief review. *J Gerontol Nurs* **2010**, *36*, 9-14, doi:10.3928/00989134-20100730-03.

28. Lim, A.Y.; Doherty, M. What of guidelines for osteoarthritis? *Int J Rheum Dis* **2011**, *14*, 136-144, doi:10.1111/j.1756-185X.2011.01609.x.

29. Woodcock, J. A difficult balance--pain management, drug safety, and the FDA. *The New England journal of medicine* **2009**, *361*, 2105-2107, doi:10.1056/NEJMp0908913.

30. Scheiman, J.M.; Sidote, D. Which NSAID for your patient with osteoarthritis? *J Fam Pract* **2010**, *59*, E1-6.

31. Howes, F.; Buchbinder, R.; Winzenberg, T.B. Opioids for osteoarthritis? Weighing benefits and risks: a Cochrane Musculoskeletal Group review. *J Fam Pract* **2011**, *60*, 206-212.

32. Vista, E.S.; Lau, C.S. What about supplements for osteoarthritis? A critical and evidenced-based review. *Int J Rheum Dis* **2011**, *14*, 152-158, doi:10.1111/j.1756-185X.2011.01619.x.

33. Busija, L.; Bridgett, L.; Williams, S.R.; Osborne, R.H.; Buchbinder, R.; March, L.; Fransen, M. Osteoarthritis. *Best Pract Res Clin Rheumatol* **2010**, *24*, 757-768, doi:10.1016/j.berh.2010.11.001.

34. Kapoor, M.; Martel-Pelletier, J.; Lajeunesse, D.; Pelletier, J.P.; Fahmi, H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol* **2011**, *7*, 33-42, doi:10.1038/nrrheum.2010.196.

35. Poulet, B.; Beier, F. Targeting oxidative stress to reduce osteoarthritis. *Arthritis research & therapy* **2016**, *18*, 32, doi:10.1186/s13075-015-0908-7.

36. Palmieri, B.; Lodi, D.; Capone, S. Osteoarthritis and degenerative joint disease: local treatment options update. *Acta Biomed* **2010**, *81*, 94-100.

37. Iannitti, T.; Lodi, D.; Palmieri, B. Intra-articular injections for the treatment of osteoarthritis: focus on the clinical use of hyaluronic acid. *Drugs R D* **2011**, *11*, 13-27, doi:10.2165/11539760-000000000-00000.

38. Huang, S.L.; Ling, P.X.; Zhang, T.M. Oral absorption of hyaluronic acid and phospholipids complexes in rats. *World journal of gastroenterology* **2007**, *13*, 945-949, doi:10.3748/wjg.v13.i6.945.

39. Bannuru, R.R.; Natov, N.S.; Dasi, U.R.; Schmid, C.H.; McAlindon, T.E. Therapeutic trajectory following intra-articular hyaluronic acid injection in knee osteoarthritis--meta-analysis. *Osteoarthritis Cartilage* **2011**, *19*, 611-619, doi:10.1016/j.joca.2010.09.014.

40. Cooper, C.; Rannou, F.; Richette, P.; Bruyere, O.; Al-Daghri, N.; Altman, R.D.; Brandi, M.L.; Basset, S.C.; Herrero-Beaumont, G.; Migliore, A., et al. Use of Intraarticular Hyaluronic Acid in the Management of Knee Osteoarthritis in Clinical Practice. *Arthrit Care Res* **2017**, *69*, 1287-1296, doi:10.1002/acr.23204.

41. Bowman, S.; Awad, M.E.; Hamrick, M.W.; Hunter, M.; Fulzele, S. Recent advances in hyaluronic acid based therapy for osteoarthritis. *Clin Transl Med* **2018**, *7*, doi:ARTN 6 10.1186/s40169-017-0180-3.

42. Ponist, S.; Drafi, F.; Kuncirova, V.; Mihalova, D.; Rackova, L.; Danisovic, L.; Ondrejickova, O.; Tumova, I.; Trunova, O.; Fedorova, T., et al. Effect of Carnosine in Experimental Arthritis and on Primary Culture Chondrocytes. *Oxid Med Cell Longev* **2016**, *2016*, 8470589, doi:10.1155/2016/8470589.

43. Fatih Aydin, A.; Kucukgergin, C.; Bingul, I.; Dogan-Ekici, I.; Dogru-Abbasoglu, S.; Uysal, M. Effect of Carnosine on Renal Function, Oxidation and Glycation Products in the Kidneys of High-Fat Diet/Streptozotocin-Induced Diabetic Rats. *Exp Clin Endocrinol Diabetes* **2017**, *125*, 282-289, doi:10.1055/s-0043-100117.

44. Hipkiss, A.R. On the enigma of carnosine's anti-ageing actions. *Exp Gerontol* **2009**, *44*, 237-242, doi:10.1016/j.exger.2008.11.001.

45. Yang, Y.; Wang, Y.; Kong, Y.; Zhang, X.; Zhang, H.; Gang, Y.; Bai, L. Carnosine Prevents Type 2 Diabetes-Induced Osteoarthritis Through the ROS/NF-kappaB Pathway. *Front Pharmacol* **2018**, *9*, 598, doi:10.3389/fphar.2018.00598.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 January 2020 doi:10.20944/preprints202001.0105.v1

Peer-reviewed version available at Appl. Sci. 2020, 10, 1324; doi:10.3390/app10041324

46. Suantawee, T.; Tantavisut, S.; Adisakwattana, S.; Tanavalee, A.; Yuktanandana, P.; Anomasiri, W.; Deepaisarnsakul, B.; Honsawek, S. Oxidative stress, vitamin e, and antioxidant capacity in knee osteoarthritis. *Journal of clinical and diagnostic research : JCDR* **2013**, *7*, 1855-1859, doi:10.7860/JCDR/2013/5802.3333.

47. Chevalier, X.; Eymard, F.; Richette, P. Biologic agents in osteoarthritis: hopes and disappointments. *Nat Rev Rheumatol* **2013**, *9*, 400-410, doi:10.1038/nrrheum.2013.44.

48. Scanzello, C.R. Chemokines and inflammation in osteoarthritis: Insights from patients and animal models. *J Orthop Res* **2017**, *35*, 735-739, doi:10.1002/jor.23471.

49. Xu, Y.K.; Ke, Y.; Wang, B.; Lin, J.H. The role of MCP-1-CCR2 ligand-receptor axis in chondrocyte degradation and disease progress in knee osteoarthritis. *Biol Res* **2015**, *48*, 64, doi:10.1186/s40659-015-0057-0.

50. Campo, G.M.; Avenoso, A.; Campo, S.; D'Ascola, A.; Ferlazzo, A.M.; Calatroni, A. The antioxidant and antifibrogenic effects of the glycosaminoglycans hyaluronic acid and chondroitin-4-sulphate in a subchronic rat model of carbon tetrachloride-induced liver fibrogenesis. *Chemico-biological interactions* **2004**, *148*, 125-138, doi:10.1016/j.cbi.2004.05.004.

51. Braga, P.C.; Dal Sasso, M.; Lattuada, N.; Greco, V.; Sibilia, V.; Zucca, E.; Stucchi, L.; Ferro, E.; Ferrucci, F. Antioxidant activity of hyaluronic acid investigated by means of chemiluminescence of equine neutrophil bursts and electron paramagnetic resonance spectroscopy. *J Vet Pharmacol Ther* **2015**, *38*, 48-54, doi:10.1111/jvp.12141.

52. Treister, R.; Honigman, L.; Lawal, O.D.; Lanier, R.K.; Katz, N.P. A deeper look at pain variability and its relationship with the placebo response: results from a randomized, double-blind, placebo-controlled clinical trial of naproxen in osteoarthritis of the knee. *Pain* **2019**, *160*, 1522-1528, doi:10.1097/j.pain.0000000000001538.