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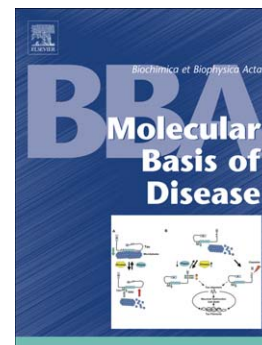
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**HYALURONAN REDUCES INFLAMMATION IN EXPERIMENTAL ARTHRITIS BY
MODULATING TLR-2 AND TLR-4 CARTILAGE EXPRESSION**

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Abstract

Previous studies have reported that low molecular mass HA and highly polymerized HA respectively elicited pro- and anti-inflammatory responses by modulating the toll-like receptor 4 (TLR-4) and the TLR-2. The activation of TLR-4 and TLR-2 mediated by collagen-induced arthritis (CIA) induces the myeloid differentiation primary response protein (MyD88) and the tumor necrosis factor receptor-associated factor 6 (TRAF6), and ends with the liberation of NF- κ B which, in turn, stimulates pro-inflammatory cytokine production. The aim of this study was to investigate the influence of high molecular weight HA at different concentrations on TLR-4 and TLR-2 modulation in CIA in mice.

Arthritis was induced in mice via intradermal injection of an emulsion containing bovine type II collagen in complete Freund's adjuvant. Mice were treated with HA intraperitoneally daily for 30 days.

CIA increased TLR-4, TLR-2, MyD88 and TRAF6 mRNA expression and the related protein in the cartilage of arthritic joints. High levels of both mRNA and related protein were also detected for tumor necrosis factor alpha (TNF- α), interleukin 1-beta (IL-1- β), interleukin-17 (IL-17), matrix metalloproteinase-13 (MMP-13) and inducible nitric oxide synthase (iNOS) in the joint of arthritic mice. HA treatment significantly limited CIA incidence and decreased all the parameters up-regulated by CIA. The improvement of biochemical parameters was also supported by histological analysis, plasma and synovial fluid HA levels.

These results suggest that the TLR-4 and TLR-2 play an important role in the arthritis mechanism and the interaction/block of HA at high molecular mass may reduce inflammation and cartilage injury.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease leading to joint inflammation, functional disability, deformities, and shortened life expectancy [1].

The hallmark of RA is synovial inflammation which directly drives extracellular matrix (ECM) degradation with consequent joint cartilage destruction and bone alterations [2]. In RA the synovial inflammation induces a disturbance in the regulation of anabolic and catabolic activities of cartilage. The end result is a net loss of cartilage with deterioration of its structural and functional properties. The factors influencing cartilage breakdown are invasion cells such as resident cells of the joint, in particular synovial fibroblasts, or activated macrophages, together with their inflammatory products such as cytokines, chemokines and proteinases including metalloproteinases (MMPs) [2-3].

Previous studies reported that pro-inflammatory cytokines produced by the synovial tissues from arthritic joints involve the activation of several TLRs [4-6]. Further investigations demonstrated that it is the TLR-4 and TLR-2 that particularly seem to be deeply involved in the mediation of the inflammatory mechanisms of arthritis and which in the end lead to synovial inflammation and the consequent cartilage erosion [7-9].

Hyaluronan (HA) is a non-sulphated major glycosaminoglycan (GAG) of the ECM that has been shown to undergo rapid degradation at inflammation sites resulting in the accumulation of lower molecular weight HA fragments [10-11]. It has been reported that low molecular weight degradation products of HA may elicit various pro-inflammatory responses, such as the activation of murine alveolar macrophages [12]. Other reports showed that low molecular weight HA oligosaccharides induce a complete and irreversible phenotypic and functional maturation of human dendritic cells, while high molecular weight HA has no such effect [13]. Of particular interest is the evidence that the activation of the TLR is not limited solely to PAMPs, but that other different molecules have also been described as alternative ligands [14]. Among these, HA is described as an interacting agent capable of exerting different effects [15-17]. Interestingly, the effect of HA on the

inflammatory response activated by TLRs, especially TLR-4 and TLR-2, appears to be related to its molecular size, i.e. high molecular weight hyaluronan has anti-inflammatory activity [18-24] while small hyaluronan fragments have pro-inflammatory activity [15-17, 25]. About the protective action of high molecular mass HA, a series of different hypotheses have been postulated by several Authors, however the exact mechanism seems to be yet unclear [18-24].

Accordingly, armed with these findings, and since in our previous *in vitro* studies we have found an involvement of TLR-4 and the consequent NF- κ B inhibition, exerted by high molecular weight HA, in chondrocytes stimulated with lipopolysaccharide (LPS) [18, 26], the aim of the present study was to investigate, using a CIA model in mice, whether the chronic administration of high molecular weight HA, as previously evaluated in CIA in rats [27-28], involves TLR-2, TLR-4 and NF- κ B modulation.

2. Materials and Methods

2.1. Animals.

Male mice DBA/J1 6-7 weeks old with a mean weight of 25-30 g were used in our study. Mice, purchased from Harlan (Correzzana, Italy), were maintained under climate-controlled conditions with a 12-h light/dark cycle. The animals were fed standard rodent chow and provided water *ad libitum*. The health status of the animal colony was monitored in accordance with Italian Veterinary Board guidelines. Mice were divided into the following groups: 1) Control (n=17); 2) HA (40 mg/kg) (n=17); 3) CIA (n=27); 4) CIA + HA (10 mg/kg) (n=23); 5) CIA +HA (20 mg/kg) (n=23); 6) CIA + HA (40 mg/kg) (n=23). Two additional set of mice (n=7 for each group) were considered in order to evaluate HA levels in plasma and synovial fluid. One set of mice was sacrificed at day 35, while the second set was sacrificed at day 50.

2.2. Materials.

High molecular weight HA (4,000 KDa) was purchased from Sigma-Aldrich Srl, (Milan, Italy). The compound was endotoxin free (endotoxin content <0.1 ng/mg). Bovine type II collagen and complete Freund's Adjuvant were obtained from MD Biosciences (Zurich, Switzerland). Mouse TNF- α (cat. IB49688), IL-1 β (cat. IB49700), IL-17 (cat. IB49699) commercial ELISA kits were provided by Immuno-Biological laboratories Inc. (Minneapolis, MN, USA). Mouse MMP-13 (cat. E0099m) and iNOS (cat. E0837Mu) commercial ELISA kits were obtained from USCN Life Science Inc. Wuhan (Wuhan, China). Rabbit TLR-4 (cat. sc-30002), TLR-2 (cat. sc-10739), MyD88 (cat. sc-11356) and TRAF6 (cat. sc-7221) polyclonal antibodies and horseradish peroxidase-labeled goat anti-rabbit antibodies (cat sc-2004) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents used were purchased from Fluka (division of Sigma-Aldrich Srl, Milan, Italy).

2.3. Induction of CIA and arthritis evaluation.

Mice were injected intradermally, at the tail base, with 100 μ L of 2.0 mg/ml type II collagen emulsion in Freund's complete adjuvant containing 2.5 mg/ml heat-killed Mycobacterium tuberculosis H37Ra. Mice were immunized a second time 7 days later.

Evaluation of joint inflammation was carried out blindly by an independent observer with no knowledge of the treatment protocol. The severity of the arthritis in each limb was graded daily on a scale of 0-4 as follows: 0, no macroscopic signs of arthritis; 1, swelling of one group of joints (i.e. wrist or ankle joints); 2, two groups of swollen joints; 3, three groups of swollen joints; 4; swelling of the entire limb. The maximum score for each mouse was 16. Clinical severity was also assessed by quantifying changes in limb volume. Measurements were performed using a dial gauge caliper. At the end of the experimental period (day 50) mice were anaesthetized with ethyl ether and then sacrificed to remove their hind limbs.

2.4. HA treatment.

On day 20, to coincide approximately with the CIA onset, the animals were randomised to receive the treatments listed above in the Animals section. HA was dissolved in saline solution (0.9% NaCl) and administered intraperitoneally using a volume of 1.0 ml/kg body weight, once a day up until day 50.

2.5 Cartilage isolation from mice joint

At the end of the experimental phase (day 50) mice were sacrificed by cervical dislocation and the whole hind limbs isolated from the body. One knee joint was conserved for histological examination, the remaining knee joint and the two paw joint were dissected and the total cartilage was separated from bone and muscular tissue, washed in ice-cold 10 mM Tris-HCl, pH 7.4, and blotted on absorbent paper. These operations were performed on cooled support refrigerated on dry ice. Whole cartilage was immediately stored on liquid nitrogen until biochemical assay .

2.6. RNA isolation, cDNA synthesis and real-time quantitative PCR amplification

Total RNA was isolated from cartilage tissue for reverse-PCR real time analysis of TLR-4, TLR-2, MyD88, TRAF6, TNF- α , IL-1 β , IL-17, MMP-13 and iNOS (RealTime PCR system, Mod. 7500, Applied Biosystems, Carlsbad, CA, USA) using an Omniscript Reagent Kit (Euroclone, West York, UK). The first strand of cDNA was synthesized from 1.0 μ g total RNA using a high capacity cDNA Archive kit (Applied Biosystems, Carlsbad, CA , USA). β -actin mRNA was used as an endogenous control to allow the relative quantification of TLR-4, TLR-2, MyD88, TRAF6, TNF- α , IL-1 β , IL-17, MMP-13 and iNOS. PCR RealTime was performed by means of ready-to-use assays (Assays on demand, Applied Biosystems, Carlsbad, CA , USA) on both targets and endogenous controls. The amplified PCR products were quantified by measuring the calculated cycle thresholds (C_T) of TLR-4, TLR-2, MyD88, TRAF6, TNF- α , IL-1 β , IL-17, MMP-13, iNOS, and β -Actin mRNA. The amounts of specific mRNA in samples were calculated by the $\Delta\Delta C_T$ method. The mean value of

normal cartilage target levels became the calibrator (one per sample) and the results are expressed as the *n*-fold difference relative to normal controls (relative expression levels).

2.7. Western blot assay of TLR-4, TLR-2, MyD88 and TRAF6 proteins

For SDS-PAGE and Western blotting, the cartilage samples were washed twice in ice-cold PBS and subsequently dissolved in SDS sample buffer (62.5 mM Tris/HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). Aliquots of whole cell protein extract (10–25 μ L/well) were separated on a mini gel (10%). The proteins were blotted onto polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK) using a semi-dry apparatus (Bio-Rad, Hercules, CA, USA). The membranes were then incubated overnight in a roller bottle with the specific diluted (1:1,000) primary antibody in 5% bovine serum albumin, 1x PBS, and 0.1% Tween 20 at 4 °C. After being washed in three stages in wash buffer (1x PBS, 0.1% Tween 20), the blots were incubated with the diluted (1:2,500) secondary polyclonal antibody (Goat anti-rabbit conjugated with peroxidase), in TBS/Tween-20 buffer, containing 5% non-fat dried milk. After 45 min of gentle shaking, the blots were washed five times in wash buffer, and the proteins, after coloration with DAB liquid substrate (Sigma-Aldrich, Milan, Italy) were made visible using a UV/visible transilluminator (EuroClone, Milan, Italy) and Kodak BioMax MR films. A densitometric analysis was also run in order to quantify each band.

2.8. NF- κ B p50/65 transcription factor assay

NF- κ B p50/65 DNA binding activity in nuclear extracts of cartilage tissue samples was evaluated in order to measure the degree of NF- κ B activation. Analysis was performed in line with the manufacturer's protocol for a commercial kit (NF- κ B p50/65 Transcription Factor Assay Colorimetric, cat. n° SGT510, Chemicon International Inc., Temecula, CA USA). Cartilage samples dissected from hind limb joints were first plotted, using an automatic potter (Mod.8533 Automatic

Potters, B. Braun, Melsungen AG, Germany), in order to isolate all cartilage cells. An aliquot of these cells ($4-5 \times 10^5$) were used for NF- κ B assay. Cytosolic and nuclear extraction was performed by lysing the cell membrane with an apposite hypotonic lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1.0% Triton, 2.5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1.0 mM β -glycerophosphate, 1.0 mM Na_3VO_4) containing protease inhibitor cocktail and tributylphosphine (TBP) as reducing agent. After centrifugation at $8,000 \times g$, the supernatant containing the cytosolic fraction was stored at -70°C , while the pellet containing the nuclear portion was then re-suspended in the apposite extraction buffer (25 mM HEPES pH 7.5, 500 mM NaCl, 1.0 mM DTT, 10 mM NaF, 10% Glycerol, 0.2% NP40, 5.0 mM MgCl_2) and the nuclei were disrupted by a series of drawing and ejecting actions. The nuclei suspension was then centrifuged at $16,000 \times g$. The supernatant fraction was the nuclear extract. After the determination of protein concentration, this extract was stored in aliquots at -80°C for the subsequent NF- κ B assay. After incubation with primary and secondary antibodies, colour development was observed following the addition of the substrate TMB/E. Finally, the absorbance of the samples was measured using a spectrophotometric microplate reader set at λ 450 nm. Values are expressed as relative optical density (OD)/mg protein.

2.9. TNF- α , IL-1 β , IL-17, MMP-13 and iNOS ELISA assay

Samples of cartilage obtained from mice hind limbs in the presence of 1.0 nM PMSF and protease inhibitor cocktail were first lysed using an opportune hypotonic lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1.0% Triton, 2.5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1.0 mM β -glycerophosphate, 1.0 mM Na_3VO_4 , 0.2% TBP) and then centrifuged at 13,000 rpm for 10 min at 4°C . The analysis of TNF- α , IL-1 β , and IL-17 was carried out using a specific commercial kit. Briefly, 50 μL of standards, samples and controls were added to each well of the coated microplate. 50 μL of each specific biotin-conjugate antibody were then added to each well. After 120 min of incubation at $20-22^\circ\text{C}$, the liquid from the wells was discarded, the wells were washed three times

and 100 μ L of Streptavidin-HRP was added. After further incubation for 60 min and washing the wells again, 100 μ L of a substrate chromogen solution was added. After 10 min incubation and the addition of 100 μ L of stop solution, the absorbance of each well was read spectrophotometrically at λ 450 nm. TNF- α IL-1 β and IL-17 values are expressed as pg/mg protein. For MMP-13 and iNOS, 100 μ L of previously diluted samples, standards and controls were added to each well of the coated microplate. After incubation for 2 hours at 37° C, the liquid of each well was discarded. After adding 100 μ L of the detection reagent A and incubation at 37° C for 1 hour, the liquid was aspirated and wells were washed with wash buffer. At this point, 100 μ L of detection reagent B was added to each well, and after a further incubation for 1 hour at 37° C the liquid of wells was first aspirated then wells were washed with wash buffer. 90 μ L of the substrate solution was added and after incubation at room temperature for 30 minutes, 50 μ L of stop solution was added. The absorbance of each well was read, within 30 minutes, spectrophotometrically at λ 450 nm. MMP-13 values are expressed as pg/mg protein, while iNOS values are expressed as U/mg protein.

2.10. HA assay.

HA analysis was performed in plasma and synovial fluid samples obtained at day 35 and at the end of the experiment (day 50). The assay was carried out using a specific enzyme-linked binding protein assay test kit (Hyaluronan ELISA kit, cat n° K-1200, Echelon Biosciences Inc., Salt Lake City, UT, USA). In brief, samples were added to a microtiter well together with an antibody-like peptide and incubated at room temperature for one h. After washing, a secondary antibody conjugated with peroxidase (HRP-conjugated) directed against this latter was added to the well and incubated at room temperature for 30 min. Subsequently, after adding a one-component substrate solution (3,3',5,5'-tetramethylbenzidine plus hydrogen peroxide) and a further incubation period (at room temperature for 30 min) the reaction was stopped with 0.36 N H₂SO₄ and the absorbance was read at 450 nm using a microplate reader (DAS srl, Rome, Italy). The concentration of HA in each

sample was determined by interpolation from a standard curve ranging from 0.0 to 800.0 ng/ml for plasma levels and from 0.0 to 100.0 µg/ml for synovial fluid levels

2.11. HA size analysis.

The HA size was evaluated on agarose gel (1.0%, 1xTAE buffer, 30 V) using a previously published method [29]. Five millilitres of each sample were first dried in a vacuum drier (CentriVap Console, Labconco, Kansas City, MO, USA) and the pellet was resuspended with 10 µl of buffered solution and then loaded per lane. Defined DNA ladder (100 bP, Stratagene, La Jolla, CA, USA) and HA molecular weight (50 kD, 100 kD, 500 kD, 2,000 kD and 3,000 kD) were also run as standards. Bands were then stained with Stains-All dye (0.005% w/v in ethanol). After electrophoresis, the gel was removed from the apparatus and was illuminated in a dark room connected up to a digital camera (mod. Coolpix 4500, Tokyo, Japan). Data were acquired and processed by the computer using a specific data acquisition program (Nikon view 5, Nikon, Tokyo, Japan).

2.12. Histology

At the end of the experimental phase, knee joints were removed from the hind limbs of the animals and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4° C for 4 hours, and then washed with 0.2 M phosphate buffer, pH 7.4. The specimens were decalcified with a ready to use decalcifier solution (Cal-Ex, Thermo Fisher Scientific, Waltham, MA, USA) for 6 hours, dehydrated in graded ethanol and embedded in a special tissue embedding media (Paraplast, McCormick Scientific, Richmond, VA, USA). Traditional histological sections (7 µM) were cut with a microtome (mod. RM 2125 RT, Leica Camera AG, Solms, Germany), stained with Ematoxilin-Eosin technique and viewed and photographed with a light microscope (mod. BH2, Olympus America Inc., Melville, NY, USA).

Cartilage destruction was measured as cartilage thickness and cartilage surface erosion in a serial sections of each knee joint. Cartilage thickness was determined as absolute value of the layer obtained in the sections and was expressed as mm. Cartilage surface erosion was determined as the amount of cartilage lost in the relation to the total cartilage area. All experiments were scored separately and independently of each other. Data were expressed as the mean of all cartilage layers measured [30].

2.13. Protein analysis

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Lab., Richmond, CA, USA) with bovine serum albumin as a standard in accordance with the published method [31].

2.14. Statistical analysis

Data are expressed as means \pm S.D. of no less than seven experiments for each test. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. The statistical significance of differences was set at $p < 0.05$.

2.15. Statement of animal care

The studies reported in this manuscript have been performed out in accordance with the Helsinki declaration and the NIH guidelines for the Care and Use of Laboratory Animals.

3. Results

3.1. Effects of HA treatment on clinical signs of CIA

On about day 20, animals began to show evidence of clinical inflammation in one or more hind paws. The first manifestation of disease was erythema of one or more ankle joints, followed by the metatarsal and interphalangeal joints. Fig. 1A reports the incidence of CIA over the 50-day study period. The initial signs of arthritis were evident at day 25 in all mice treated with CIA. In the CIA-group untreated with HA, the percentage of mice developing arthritis was 98% at day 25 and the same percentage was maintained up until the end of the experiment. Treatment with HA significantly attenuated the development of CIA: 15% with the dose of 10 mg/kg; 27% with the dose of 20 mg/kg and, 43% with the dose of 40 mg/kg (Fig.1A).

The end of the experiment is presented in Fig. 1B which shows the final stage of the disease, as assessed by the arthritis evaluation scale. By day 25 all animals showed evidence of the disease, predominantly in the hindpaws. The disease progressed continuously with joint recruitment following an identical pattern: tarsal, metatarsophalangeal followed by interphalangeal. The interphalangeal joints were never affected alone, and inflammation in these joints was invariably associated with inflammation in the tarsal joint. The arthritis score in CIA mice was progressive from day 25 and reached an arthritis index score of about 12.7 in the final week. The same changes were observed in the hind paw diameter of CIA animals. Administration of HA significantly attenuated all clinical signs of arthritis: a.i.= 8.2 with the dose of 10 mg/kg a.i.= 6.7 with the dose of 20 mg/kg and a.i.=5.4 with the dose of 40 mg/kg (Fig.1B).

3.2. TLR-4, MYD88 and TRAF-6 mRNA expression and Western blot analysis.

TLR-4, TLR-2, MYD88 and TRAF-6 (Figs. 2, 3, 4 and 5) mRNA evaluation (panels A of each Figure) and Western blot analysis with densitometric evaluation (Figs. 2, 3, 4 and 5, panels B and C of each Figure) were assayed in order to estimate the degree of TLR-4 and TLR-2 activation and the consequent cell signalling pathway booster that culminates in NF- κ B factor activation. Very low

gene expression and the related protein production of TLR-4, TLR-2, MYD88 and TRAF-6 were obtained in non-arthritic mice (CTRL and CTRL+HA). In contrast, a marked increase in the expression and protein synthesis of the TLR-4 and TLR-2 and their signal mediators MYD88 and TRAF-6 were found in the cartilage joint of arthritic mice. Both TLR-4 and TLR-2 were highly expressed and this means that both receptors were involved in this pathology.

The treatment with high molecular weight HA for 30 days produced a significantly reduction in the activation of both receptors and their signal mediator levels in CIA-treated mice. As reported in Figs. 2, 3, 4 and 5, HA treatment decreased TLR-4, TLR-2, MYD88 and TRAF-6 expression and protein synthesis at all concentrations used in a dose-dependent manner.

3.3. *NF- κ B* activation.

Fig. 6 shows the changes in the NF- κ B p50/p65 heterodimer translocation over the course of the experiment. NF- κ B DNA binding was present at very low levels in the joint cartilage of mice not subject to CIA. In contrast, arthritis induced massive NF- κ B translocation into the nucleus of the damaged cartilage cells; the chronic treatment of CIA-mice with HA at different concentrations reduced NF- κ B activation in a dose-dependent manner thereby confirming the involvement of TLR-4, TLR-2, MYD88 and TRAF-6 parameters

3.4 *TNF- α* , *IL-1 β* , *IL-17*, *MMP-13* and *iNOS* mRNA expression and Western blot analysis.

TNF- α , IL-1 β , IL-17, MMP-13 and iNOS were assayed because of their active role in inflammation and cartilage destruction. (Figs. 7, 8, 9, 10 and 11). Their mRNA evaluation (panel A of each Figure) and protein concentration (panel B of each Figure) showed that the mRNA of these inflammatory mediators was not stimulated in the joint cartilage of mice not subject to CIA and protein level was also very low. However, in the mice subjected to CIA, TNF- α , IL-1 β , IL-17, MMP-13 and iNOS expression and the related protein concentration were significantly up-regulated. The administration of high molecular weight HA was able to reduce this increment in

mRNA expression at all doses. As reported (Figs. 7, 8, 9, 10, and 11; panels A and B of each Figure), the increase in mRNA expression of TNF- α , IL-1 β , IL-17, MMP-13 and iNOS in arthritic mice correlated well with the increment in protein synthesis. This correlation was also maintained in mice treated with HA. In fact, the reduction in mRNA expression resulted in a similar diminution in protein formation.

3.5. *Histological analysis*

Fig. 12 reports a representative joint histopathology of all the experimental groups. Panels A and B show a knee joint section from mice not subject to CIA. The fully stained cartilage layers indicate no cartilage damage or erosion. Panel C presents a joint section from a CIA-treated mouse. This clearly shows the severe knee joint inflammation and loss of Hematoxylin-Eosin staining of the cartilage layers, as well as the complete disruption of the original cartilage architecture (indicated by triangles). In panels D, E, and F a representative selection of knee joint sections from mice receiving CIA and HA treatment shows that a gradual reduction in joint damage and cartilage erosion occurred. As indicated by arrows HA administration reduced cartilage thickness and jagged margins (Fig. 12).

We also quantified cartilage thickness and surface erosion in the same sections (Fig. 13, panels A and B). In control groups no loss of cartilage was seen. On the contrary, mice with arthritis showed a massive reduction on cartilage thickness and increase in surface erosion; the treatment of CIA-mice with HA, at different doses, was able to prevent significantly cartilage loss.

3.6. *Plasma and synovial HA levels*

HA concentrations were measured in control and CIA mice HA treated/untreated in order to compare the protective effect associated with enhanced HA plasma and synovial fluid levels and biochemical and histological parameters (Fig. 14, panels A, B, C, and D). Consistent with previous studies, plasma and synovial fluid levels of HA were significantly higher in mice underwent to CIA

and untreated with HA with respect CTRL-mice. The treatment with HA was able to increase plasma and synovial fluid HA levels both at 35 days (15 days treatment) and at 50 days (30 days treatment). The HA increase was also significant both in CIA-mice and in CTRL-mice treated with HA (Fig. 14, panels A, B, C, and D). Comparison of HA levels in mice treated with CIA suggested a possible trend for positive correlation with biochemical and histological parameters as well as with the HA doses chronically administered.

3.7. HA molecular weight.

Fig. 15 shows the detection of HA fragment size in synovial fluid at the end of the experiment. CTRL mice presented a comparable amounts of HA at both low and high molecular weight. On the contrary CTRL mice treated with HA presented an increase in HA content at high molecular weight. CIA mice mainly presented high percentage of low molecular weight HA with respect to high molecular weight HA, while the treatment of CIA mice, as well as CTRL mice, was particularly able to increase the content of HA at high molecular weight. (Fig15). The increase of HA at high molecular weight was positively correlated with the corresponding HA dose chronically administered, while the increase of HA at low molecular weight could be associated with the increase in inflammation found in CIA mice untreated with HA.

4. Discussion

Chronic inflammation is a significant factor in the pathophysiology of many forms of joint disease. Both the most commonly diagnosed arthritic conditions, RA and osteoarthritis (OA), are inflammatory diseases of the joints and progressive loss of cartilage is a characteristic feature of these diseases. Therefore, chondroprotection of the cartilage is one of the most important goals to achieve in this field. It is acknowledged that cartilage homeostasis results from the balance between its anabolic and its catabolic rates. Cartilage depletion occurs in RA through both the breakdown of

the matrix and the reduced synthesis of its macromolecular components. The ongoing challenge is to define the catabolic triggers of articular chondrocytes in the pathogenesis of cartilage degradation in various arthritic diseases. Previous studies showed that TLRs such as TLR-2 and TLR-4 are expressed in the synovium of patients with longstanding RA [8, 32-33]. When TLRs (except TLR-3) recognize PAMPs, the myeloid differentiation primary response protein (MyD88) binds to the Toll/IL-1 receptor (TIR) domain of TLRs, which triggers the intracellular interleukin-1 receptor (IL-1R) family signalling cascade. The activation of nuclear factor kappaB (NF- κ B) and mitogen-associated protein kinase (MAPK) cascades involves a signalling complex that contains MyD88, IL-1R-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF-6) [34-35]. Lastly, transcription is initiated to express several pro-inflammatory cytokines and effector cytokines, such as interferon-alpha/beta (IFN- α/β), interleukin-6 (IL-6), interleukin-1beta (IL-1 β), and tumor necrosis factor-alpha (TNF- α) or other inflammatory molecules, such as nitric oxide (NO) produced by the inducible nitric oxide synthetase (iNOS), reactive oxygen species (ROS) and metalloproteinases (MMPs) [36-37].

Considering the role of TLRs as a critical link between the innate and the adaptative immune response, it is plausible to hypothesize that continuous activation or dysregulation of TLR signalling might contribute to the pathogenesis of autoimmune diseases such as RA or OA. In fact, stimulation of TLR-2 and TLR-4 pathways in synovial fibroblasts obtained from patients with RA leads to translocation of NF- κ B, and secretion of pro-inflammatory cytokines, MMPs and other chemokines [8, 32-33]. Chondrocytes respond to the pro-inflammatory stimuli by increasing the production of proteinases, prostaglandins, and nitrogen oxide (NO) [38]. In experimental models of arthritis, TLR ligands are used repeatedly to induce the disease in susceptible animals; for instance, intra-articular injection of streptococcal cell wall fragments, or double-stranded RNA, which mainly signal through TLR-2, and TLR-3, respectively, can induce arthritis [39-40]. Furthermore, LPS from the outer membrane of gram-negative bacteria, signalling through TLR-4, is used extensively to aggravate or reactivate arthritis in distinct animal models [41-42]. It was previously reported that

TLRs can also be activated by several endogenous ligands that are released from stressed or damaged host tissue. In this context, TLR-4 can recognize extracellular matrix components such as heparan sulphate and extra domain A of fibronectin [43-44] and both TLR-2 and TLR-4 can recognize the matrix component biglycan [45]. TLR activation by these self antigens can potentially promote the development of autoimmune diseases. On the contrary, inhibiting TLR activation seems to be an important strategy to reduce cellular injury and it may reduce inflammation in arthritis in particular [46-47].

HA is a component of the ECM in various tissues, and is essential for matrix assembly and fluid viscosity in cartilage. HA has been reported to exhibit cartilage-protective effects, including stimulation of chondrocyte proliferation, production of cartilage matrix, and inhibition of protease [48], it also seems to be a potent inflammatory agent [15-88, 25].

This double role played by HA seems to be due to its molecular mass, and its effects seem, in part, to be related to its interaction with TLR-4 [15-18].

In a previous experiment we started HA administration two weeks after the arthritic signs were well developed, and continued, daily, for 30 days (unpublished data). Unfortunately, although a slight significant protection was seen, mainly by the histological evaluation, the reduction of TLR-4 and TLR-2, and other inflammatory intermediates such as TNF- α , IL-6, MMP-13, and iNOS produced not evident effects. This could be explained by the fact that when the disease is well-established, different mechanism, other than TLRs, could be involved in the chronic inflammation, and therefore the block/modulation of these receptors, responsible of the early phase of inflammation, may assume a secondary role in the chronic inflammation, typical of arthritis. On the contrary, HA treatment of mice at onset of CIA development may contrast the early mechanisms responsible of inflammation that then culminate with cartilage/bone erosion and chronic inflammation. Therefore, high molecular weight HA seems to reduce arthritis at early stage of development by blocking the initiators of the inflammatory response, while, by this same way, may not when disease is fully developed. In this context the large use of HA, by joint injection, in human RA and OA may be also

considered, with good results and mainly pain and swelling reduction [49]. In this case the mechanism could be different, as the local injection may provide a high HA concentration to the inflamed joints. In addition, as HA binds large amounts of H₂O, the joint amelioration may be due especially to its rheological/fluidising properties than to a direct interaction with receptor or other protein structures, although other specific unknown interactions couldn't excluded.

In fact, intra-articular treatment with HA and hylans has become more widely accepted in the range of therapies to combat OA and RA pain [49]. HA is directly responsible for the viscoelastic properties of synovial fluid. Thus, it seems the goal of intra-articular therapy with HA is to help replace synovial fluid that has lost its viscoelastic properties. Although exogenous HA therapy may exert this effect, the effective protective action of HA is not fully understood.

In the present study, the treatment of arthritic mice with different doses of high molecular weight HA was able to reduce inflammation and cartilage erosion induced by CIA, as also showed in our previous experiments on different types of animal [27-28]. This study examined the effects of the prolonged intra-peritoneal administration of high molecular weight HA, at different molecular concentrations, on TLR-2 and TLR-4 modulation in arthritic mice. Our findings suggest that both receptors were involved in this experimental disease and that HA reduced TLR-2 and TLR-4 expression and the inflammatory mechanism primed by their activation. Indeed, the data obtained show that the HA was able to reduce the expression of the two receptors, their intermediates whereby NF- κ B is activated, and a series of mediators of inflammation and cartilage degradation. The effects were well substantiated by the dose-dependent efficacy exerted by HA. In CIA-treated mice, HA was able to reduce not only the expression of TLR-2 and TLR4, MyD88 and TRAF6 but also NF- κ B activation and the increment in pro-inflammatory cytokines TNF- α , IL-1 β and IL-17, MMP-13 and iNOs. These findings were further confirmed by the histological results, where cartilage degradation was limited by HA treatment. A further support to these data can be given from the analysis of HA levels in plasma and synovial fluid that well correlated with biochemical and histological parameters. The increment in synovial fluid of HA at high molecular weight in

mice chronically administered with HA confirm the protective role exerted by this polymer in modulating TLR-2 and TLR-4

The interaction of cells with the surrounding ECM is fundamental in many physiological and pathological mechanisms. Proteoglycans may influence cell behaviour through binding events mediated by their GAG chains. The specificity of protein-GAG interactions is governed by the ionic attractions of sulphate and carboxylate groups of GAGs towards the basic amino acid residues on the protein as well as by the optimal structural fit of the GAG chain into the protein binding site [50]. The binding affinity of the interaction depends on the ability of the oligosaccharide sequence to provide an optimal charge and surface with the protein [51]. It has been demonstrated that the interaction of HA degradation products with TLR-2 and TLR-4 provides signals to initiate inflammation after non-infectious lung injury, whereas TLR-2 and TLR-4 serve to maintain epithelial cell integrity and tissue repair by sensing native high-molecular-mass HA [15]. Other data support the idea that a balance between low molecular weight HA and high molecular weight HA, by engaging TLR-2, may control the activation of the innate immune response in situations of tissue damage and danger [51]. In fact, in the event of tissue destruction, high molecular weight HA is broken down into low molecular weight HA species which have the ability to promote inflammation by inducing the release of reactive oxygen species, such as NO, cytokines, such as TNF- α and IL-1 β , and destructive enzymes, such as MMPs and by facilitating the recruitment of CD44⁺ leukocytes [17, 52]. While high molecular weight HA maintains homeostasis and potentially down-regulates inflammation, the generation of low molecular weight HA may act as an endogenous danger signal, leading to the activation of both innate and acquired immunity. The fact that a lack of low molecular weight HA clearance leads to excess damage, whereas the over-expression of high molecular weight HA is protective in the non-infectious lung injury model supports this hypothesis [11, 15, 53]. Although PAMPs alert the immune system to exogenous pathogens and molecules like uric acid and others signal necrotic cell death, low molecular weight HA heralds a breach of barriers and the destruction of tissue integrity [54]. In this way, low

molecular weight HA-induced TLR signalling might activate the immune response before the development of an established infection or necrotic cell death. Although there is a constant turnover of high molecular weight HA in the day-to-day maintenance of a normal matrix, it is rapidly degraded into very small non-biologically active fragments which are quickly cleared by the liver [10]. Therefore, the positive modulatory effect exerted by high molecular weight HA on all the parameters considered could be due to its efficiency in binding protein structures, such as TLR-2 and TLR4, thereby exerting a block that prevents receptor stimulation by the specific ligands produced during arthritis. We previously reported that medium molecular weight HA does not exert any protective/inflammatory action on chondrocytes stimulated with LPS [18]. This could be explained by the hypothesis that, as its structure is probably unable to mask the TLRs studied or to act as a TLR agonist, it is therefore not able to reduce or to stimulate production of pro-inflammatory and degradative mediators. We suggest that the number of interaction sites available in the HA structures may play the key role in HA modulatory activity during the inflammatory mechanism. From these findings we may thus conclude that high molecular weight HA via its polymerized structure may mask TLR-2 and TLR-4 thereby preventing the stimulation of these receptor by other agents and the consequent activation of NF- κ B that culminates in the transcription of the detrimental mediators responsible for arthritic injury.

However, further studies are needed to fully confirm these hypotheses.

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5. References

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6. Figure legends

Fig. 1

Effect of HA treatment on the development (A) and progression (B) of arthritis at the end of experiment (day 50). Evaluation was performed on day 50. Values are the mean \pm S.D. of seven experiments. $^{\circ}p < 0.001$ vs Control; $*p < 0.005$ and $**p < 0.001$ vs CIA.

Fig. 2

Effect of HA treatment on articular cartilage TLR-4 mRNA expression (A) and related protein production (B, C) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as both Densitometric analysis (C) and Western Blot analysis (B) for the TLR-4 protein levels. $^{\circ}p < 0.001$ vs Control; $*p < 0.01$, $**p < 0.005$ and $***p < 0.001$ vs CIA.

Fig. 3

Effect of HA treatment on articular cartilage TLR-2 mRNA expression (A) and related protein production (B, C) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as both Densitometric analysis (C) and Western Blot analysis (B) for the TLR-2 protein levels. $^{\circ}p < 0.001$ vs Control; $*p < 0.01$, $**p < 0.005$ and $***p < 0.001$ vs CIA.

Fig. 4

Effect of HA treatment on articular cartilage MyD88 mRNA expression (A) and related protein production (B, C) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as both Densitometric analysis (C) and Western Blot analysis (B) for MyD88 protein levels. $^{\circ}p < 0.001$ vs Control; $*p < 0.01$, $**p < 0.005$ and $***p < 0.001$ vs CIA.

Fig. 5

Effect of HA treatment on articular cartilage TRAF6 mRNA expression (A) and related protein production (B, C) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as both Densitometric analysis (C) and Western Blot analysis (B) for the TRAF6 protein levels. $^{\circ}$ p<0.001 vs Control; *p<0.01, **p<0.005 and ***p<0.001 vs CIA.

Fig. 6

Effect of HA treatment on articular cartilage NF- κ B p50/65 transcription factor DNA binding activity in joints of mice with CIA. White bars represent the p/50 subunit, black bars represent the p/65 subunit. Values are the mean \pm S.D. of seven experiments and are expressed as Optical Density at λ 450 nm/mg protein of nuclear extract. $^{\circ}$ p<0.001 vs Control; *p<0.005 and **p<0.001 vs CIA.

Fig.7

Effect of HA treatment on articular cartilage TNF- α mRNA expression (A) and related protein production (B) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as pg/mg protein (B) for the TNF- α protein levels. $^{\circ}$ p<0.001 vs Control; *p<0.01, **p<0.005 and ***p<0.001 vs CIA.

Fig. 8

Effect of HA treatment on articular cartilage IL1- β mRNA expression (A) and related protein production (B) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are

expressed as the *n*-fold increase with respect to the Control (A) and as pg/mg protein (B) for the IL1- β protein levels. $^{\circ}p < 0.001$ vs Control; $*p < 0.01$, $**p < 0.005$ and $***p < 0.001$ vs CIA.

Fig. 9

Effect of HA treatment on articular cartilage IL-17 mRNA expression (A) and related protein production (B) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as pg/mg protein (B) for the IL-17 protein levels. $^{\circ}p < 0.001$ vs Control; $*p < 0.01$, $**p < 0.005$ and $***p < 0.001$ vs CIA.

Fig. 10

Effect of HA treatment on articular cartilage MMP-13 mRNA expression (A) and related protein production (B) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as pg/mg protein (B) for the MMP-13 protein levels. $^{\circ}p < 0.001$ vs Control; $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$ vs CIA.

Fig. 11

Effect of HA treatment on articular cartilage iNOS mRNA expression (A) and related protein production (B) in joints of mice with CIA. Values are the mean \pm S.D. of seven experiments and are expressed as the *n*-fold increase with respect to the Control (A) and as pg/mg protein (B) for the iNOS protein levels. $^{\circ}p < 0.001$ vs Control; $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$ vs CIA.

Fig. 12

Effect of HA on matrix proteoglycan loss and erosion in joint cartilage. Evaluation was performed at the end of the experiment, on day 50. Ematoxilin-Eosin staining (magnification 2080 X).

Fig. 13

Effect of HA on cartilage thickness (A) and surface erosion (B). Evaluation was performed at the end of the experiment, on day 50. Values are the mean \pm S.D. of seven experiments and are expressed in mm (A) and as % of cartilage destroyed (B). $^{\circ}p < 0.001$ vs Control; $*p < 0.001$ vs CIA. Average diameter of the chondrocyte = 13.31 μm ; 1.0 mm = 16.6 μm .

Fig.14

Effect of HA treatment on plasma and synovial fluid HA levels in Control and CIA mice after 15 day treatment and at the end of the experiment (day 50). Values, expressed as ng/ml for plasma HA and as $\mu\text{g}/\text{mg}$ protein for synovial fluid, are the mean \pm S.D. of seven experiments. $\#p < 0.01$ vs Control; $^{\circ}p < 0.001$ vs Control; $*p < 0.01$, $**p < 0.005$ and $***p < 0.001$ vs CIA.

Fig.15

Percentage of high and low molecular weight HA in synovial fluid at the end of experiment (day 50). White bars indicate HA at high molecular weight (more than 500 kD); black bars indicate HA at low molecular weight (from 50 to 500 kD). Values are the mean \pm S.D. of seven experiments. $^{\circ}p < 0.001$ vs Control; $\$p < 0.001$ vs Control; $*p < 0.005$ and $**p < 0.001$ vs CIA.

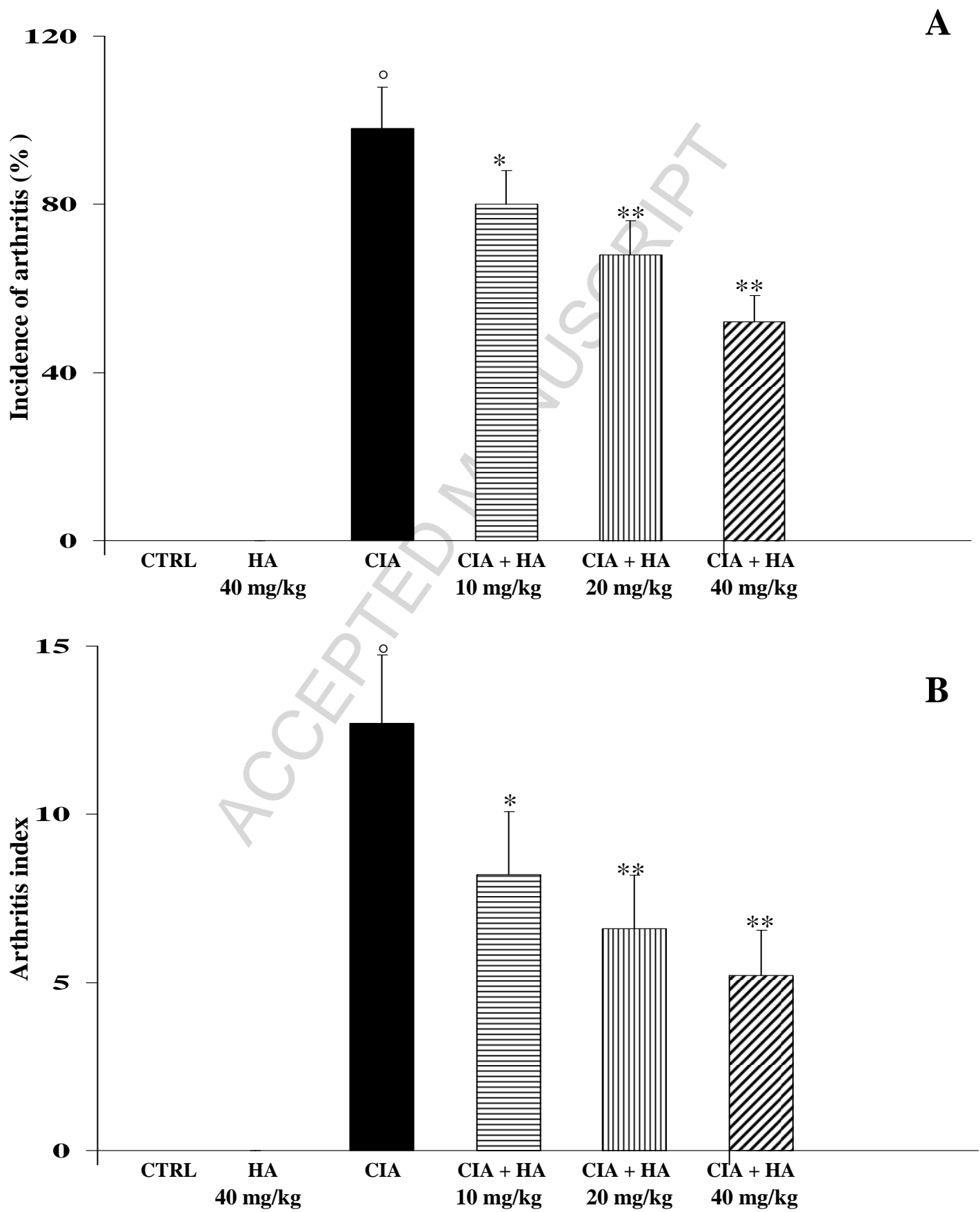


Fig. 1

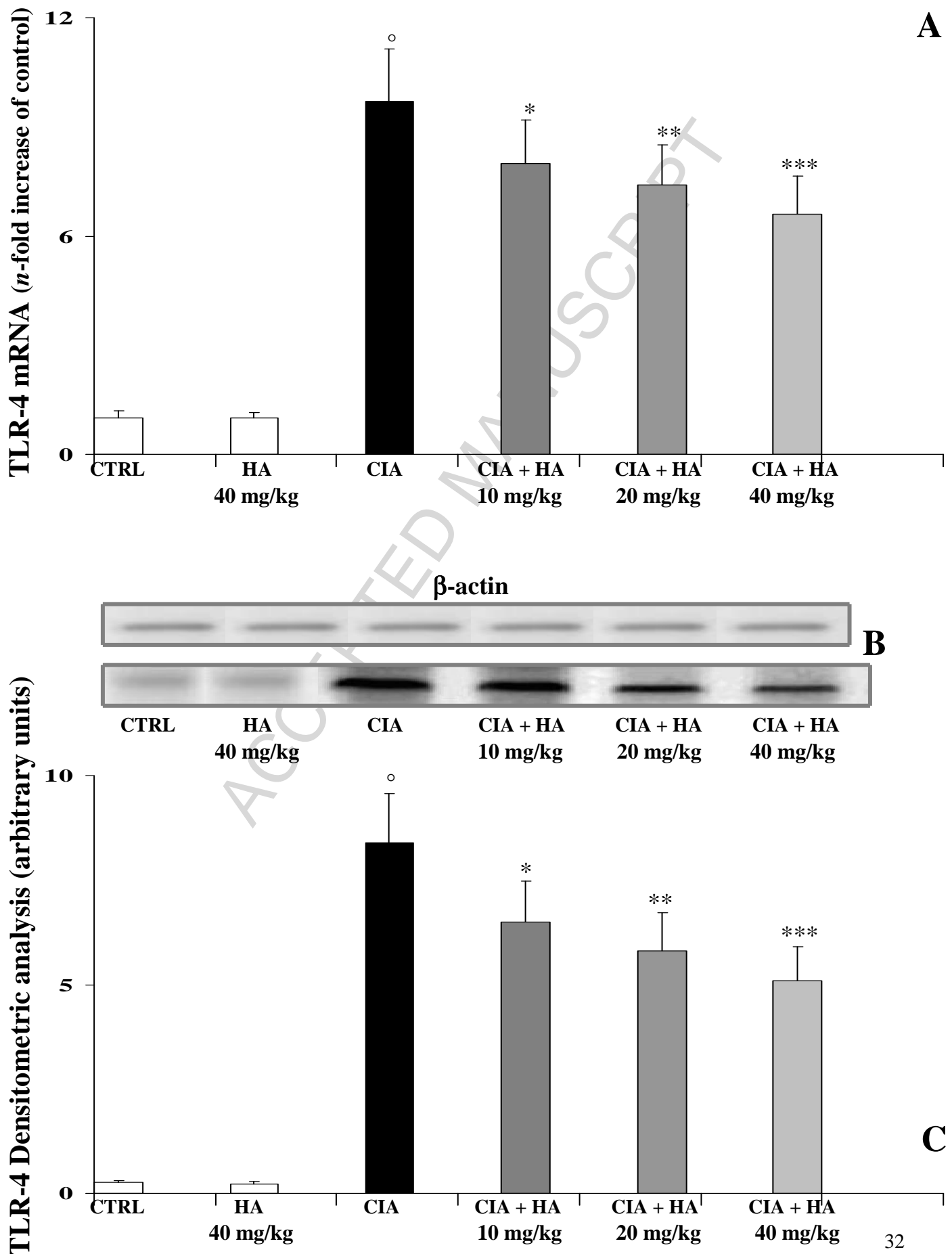


Fig. 2

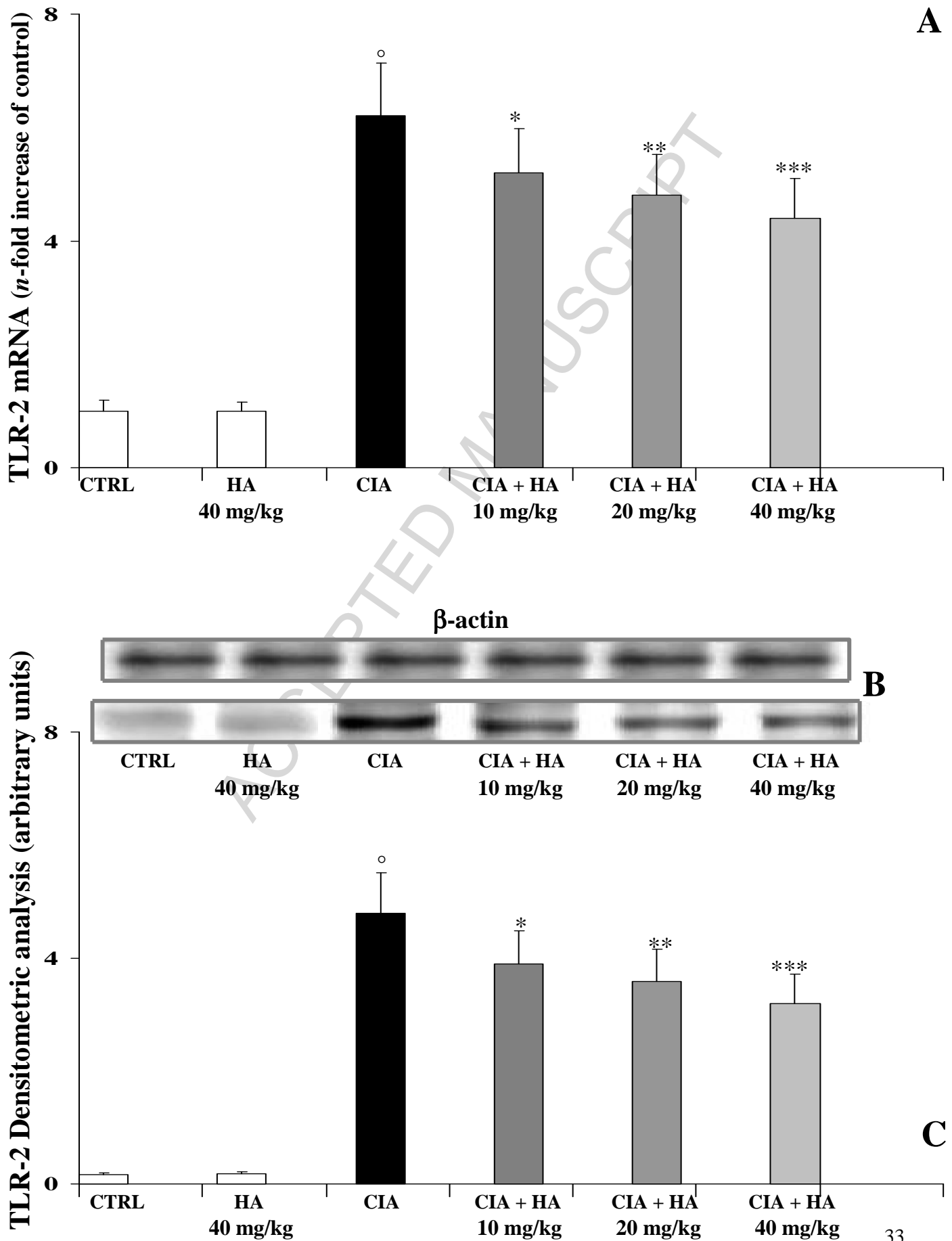


Fig. 3

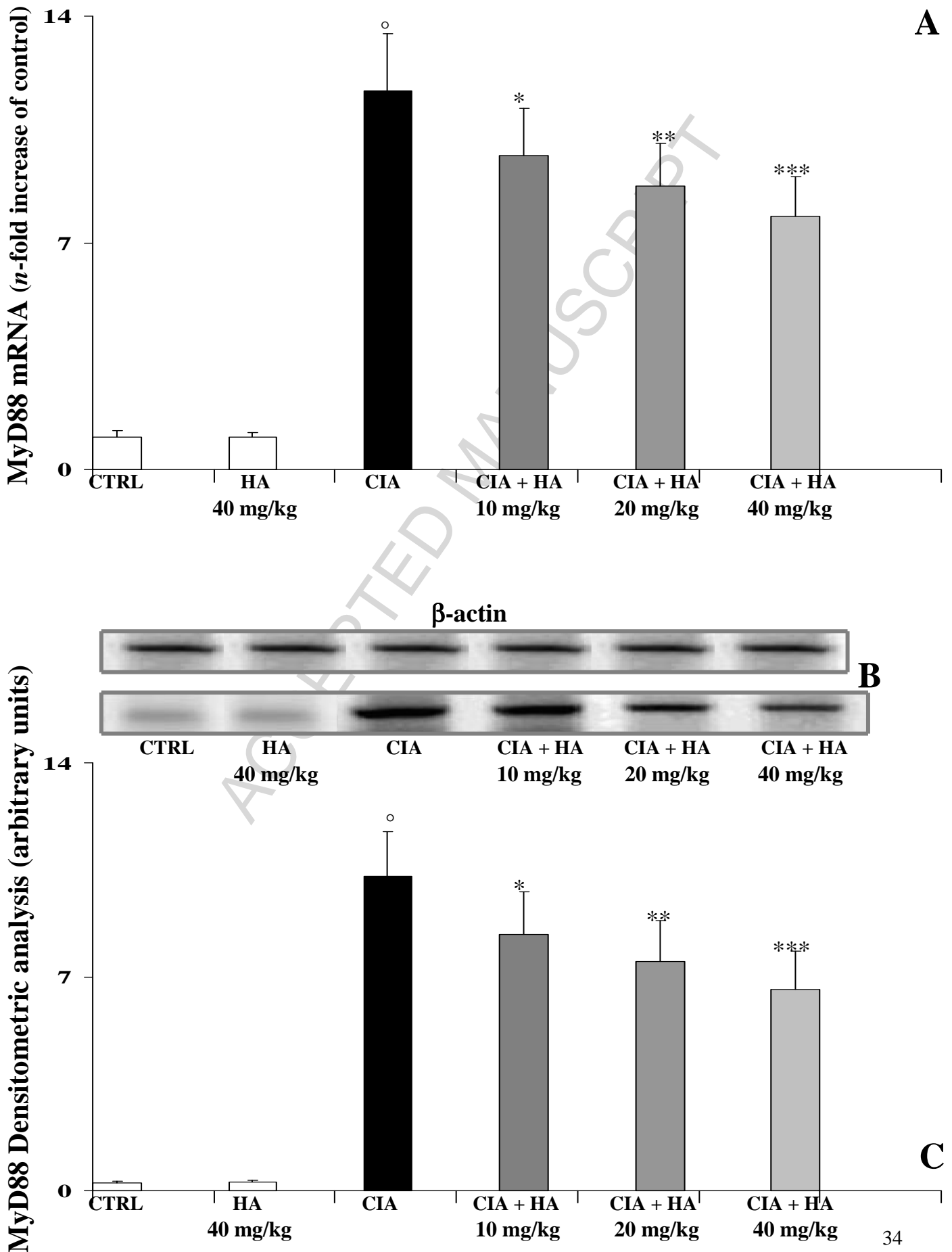


Fig. 4

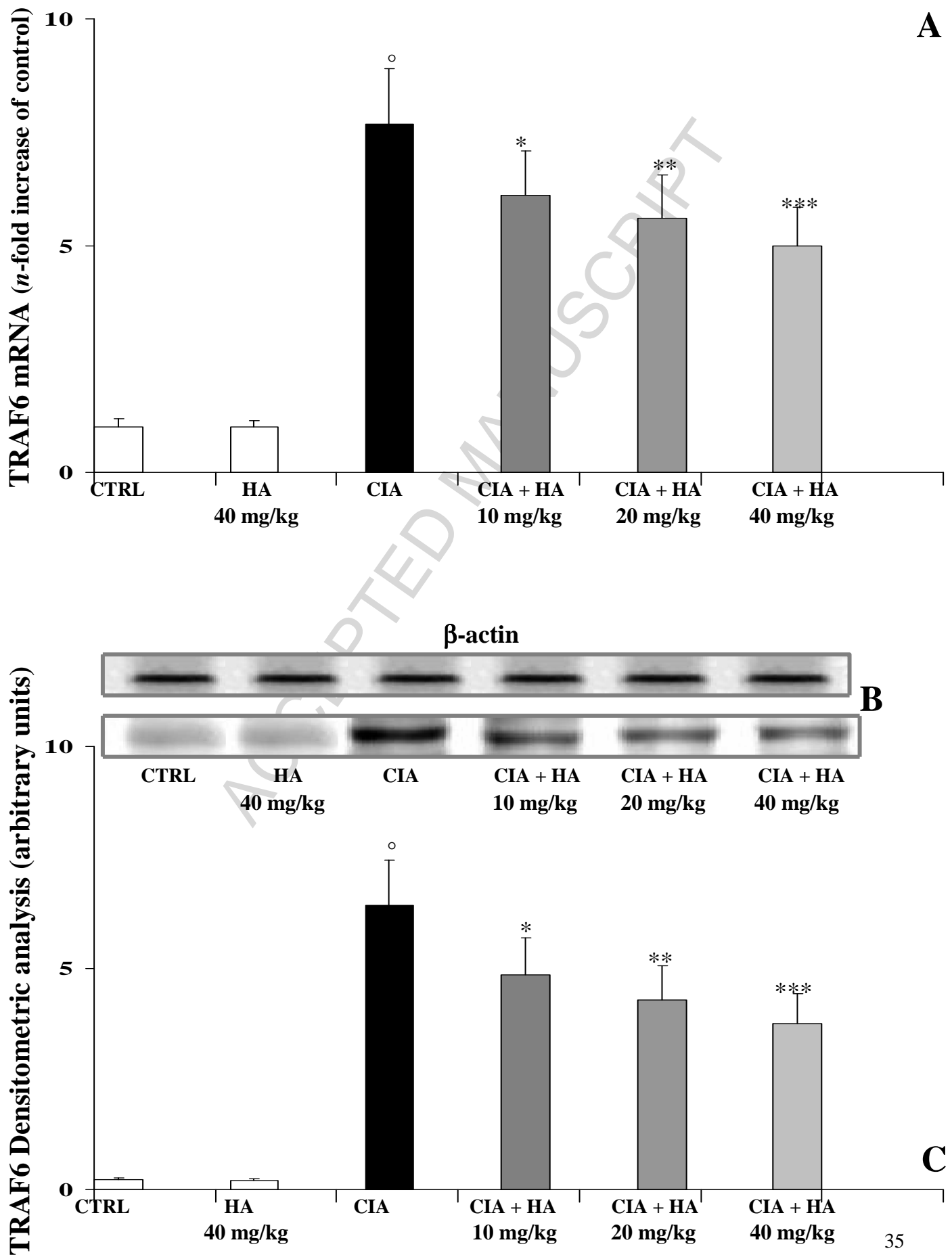


Fig. 5

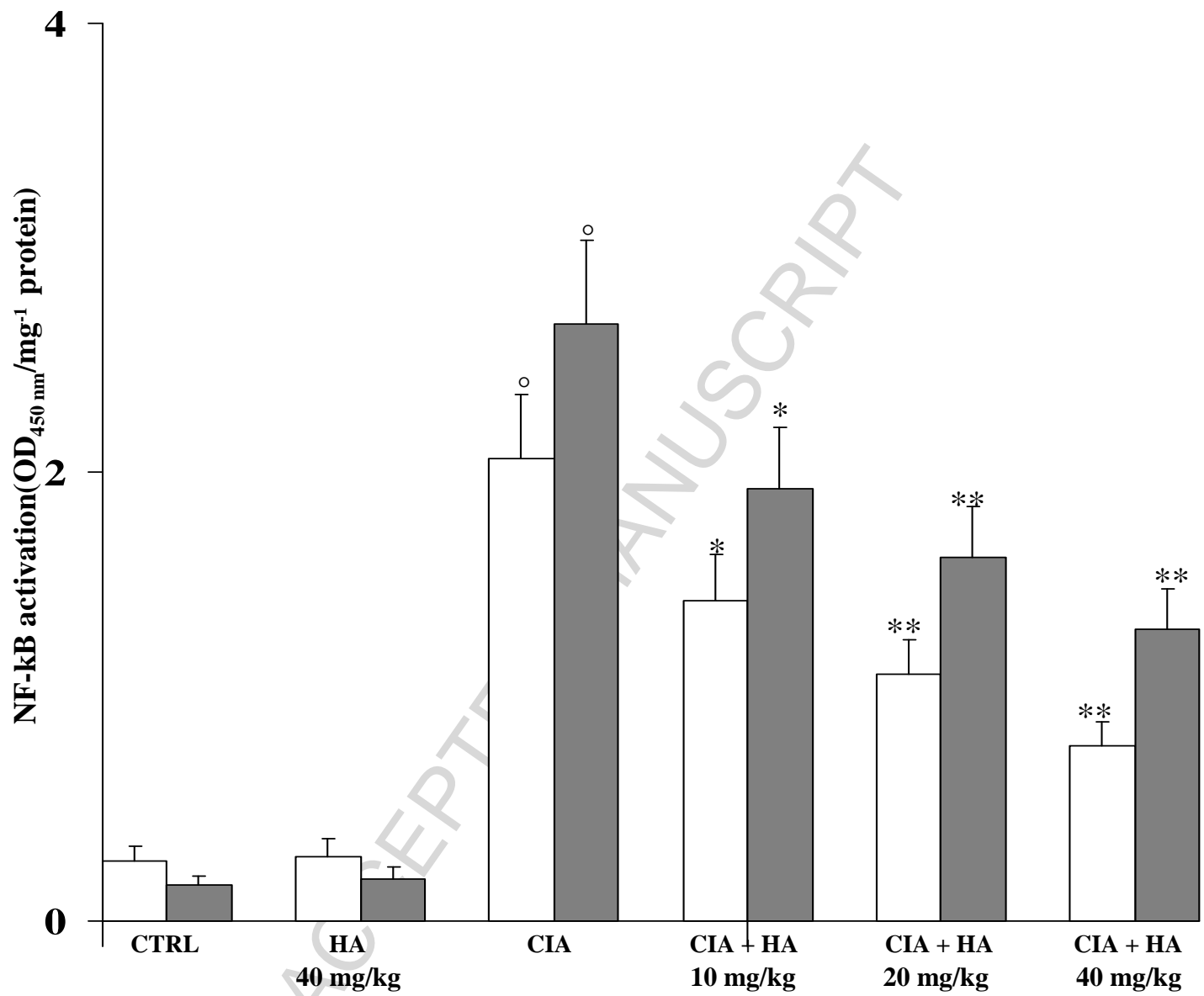


Fig. 6

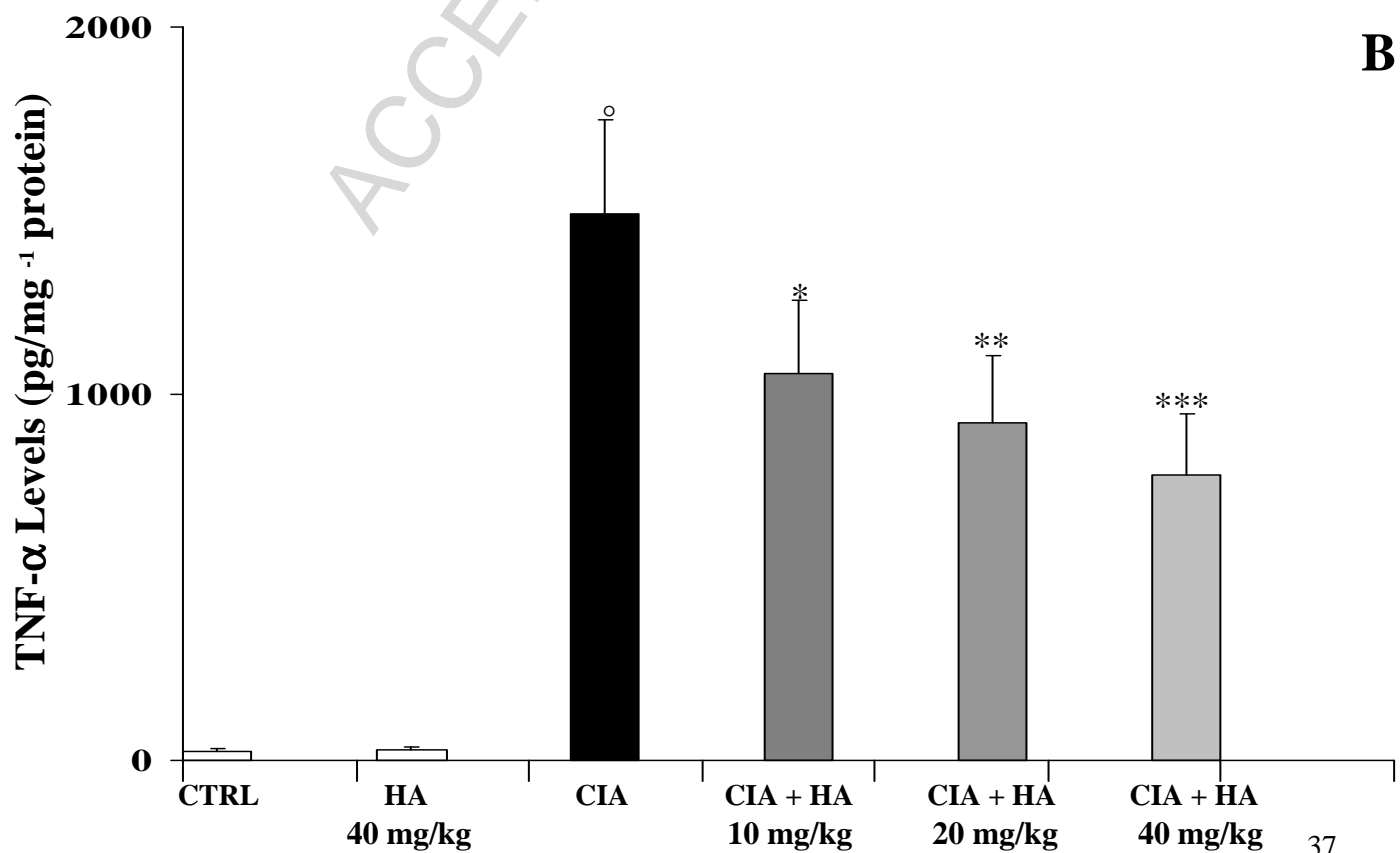
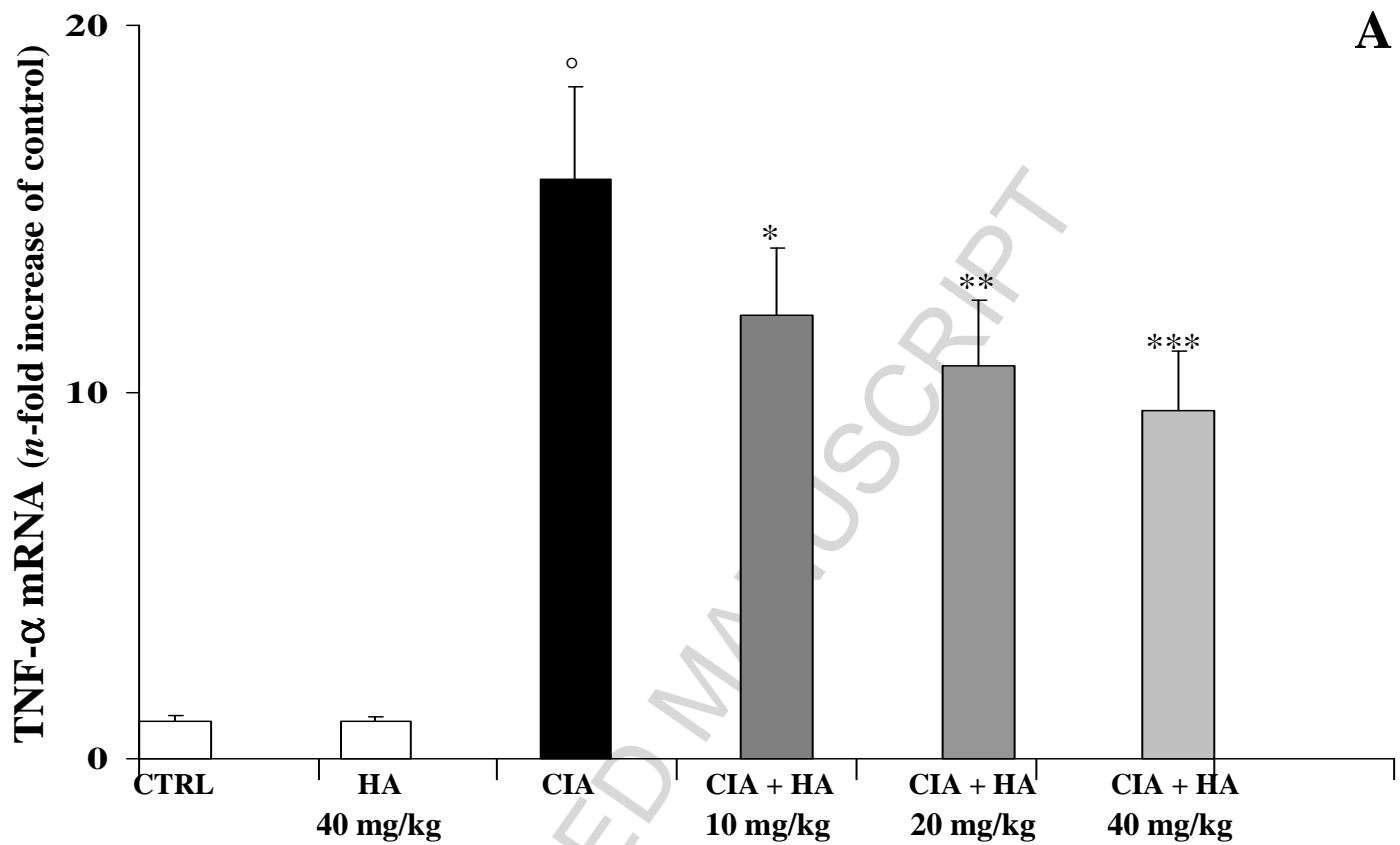


Fig. 7

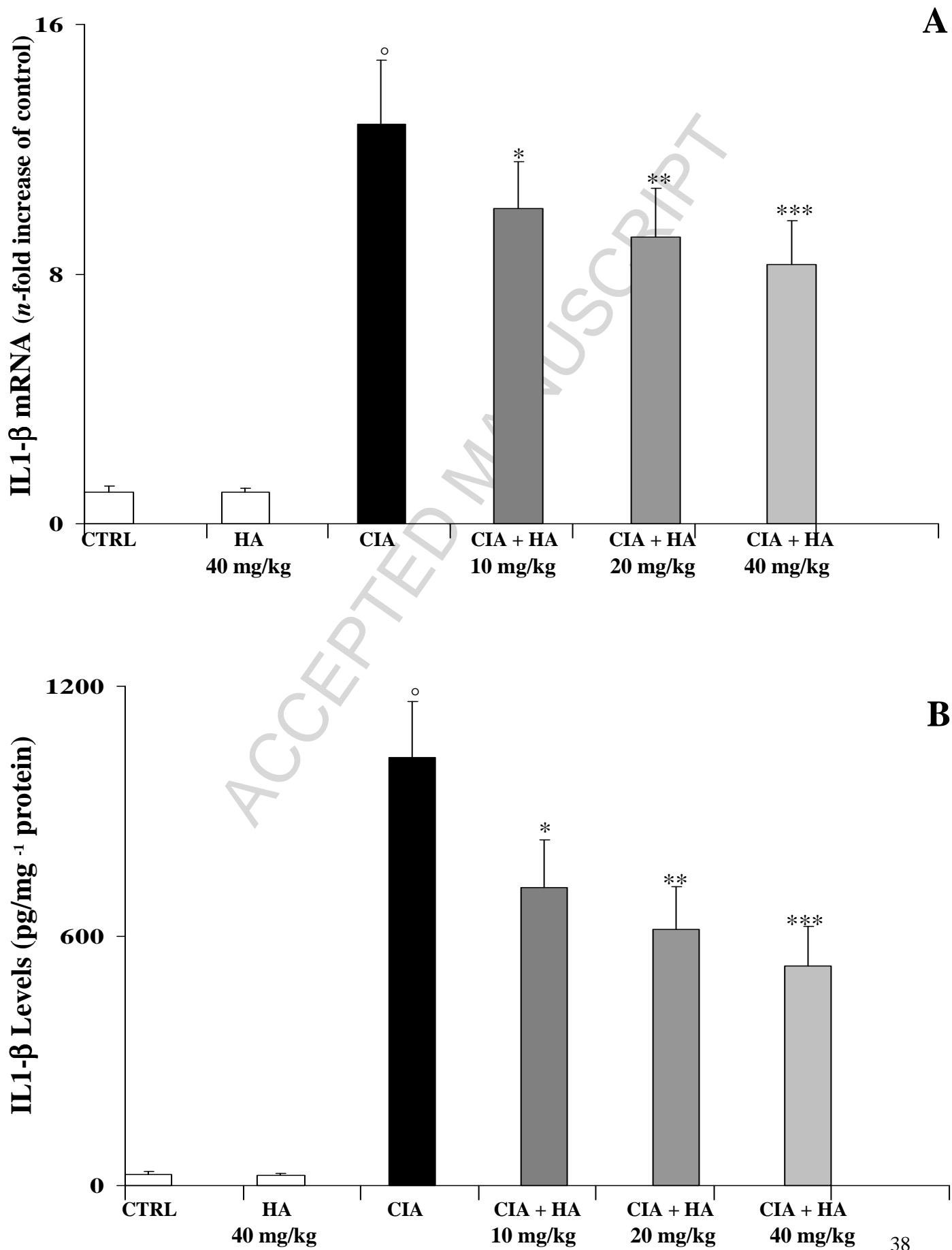


Fig. 8

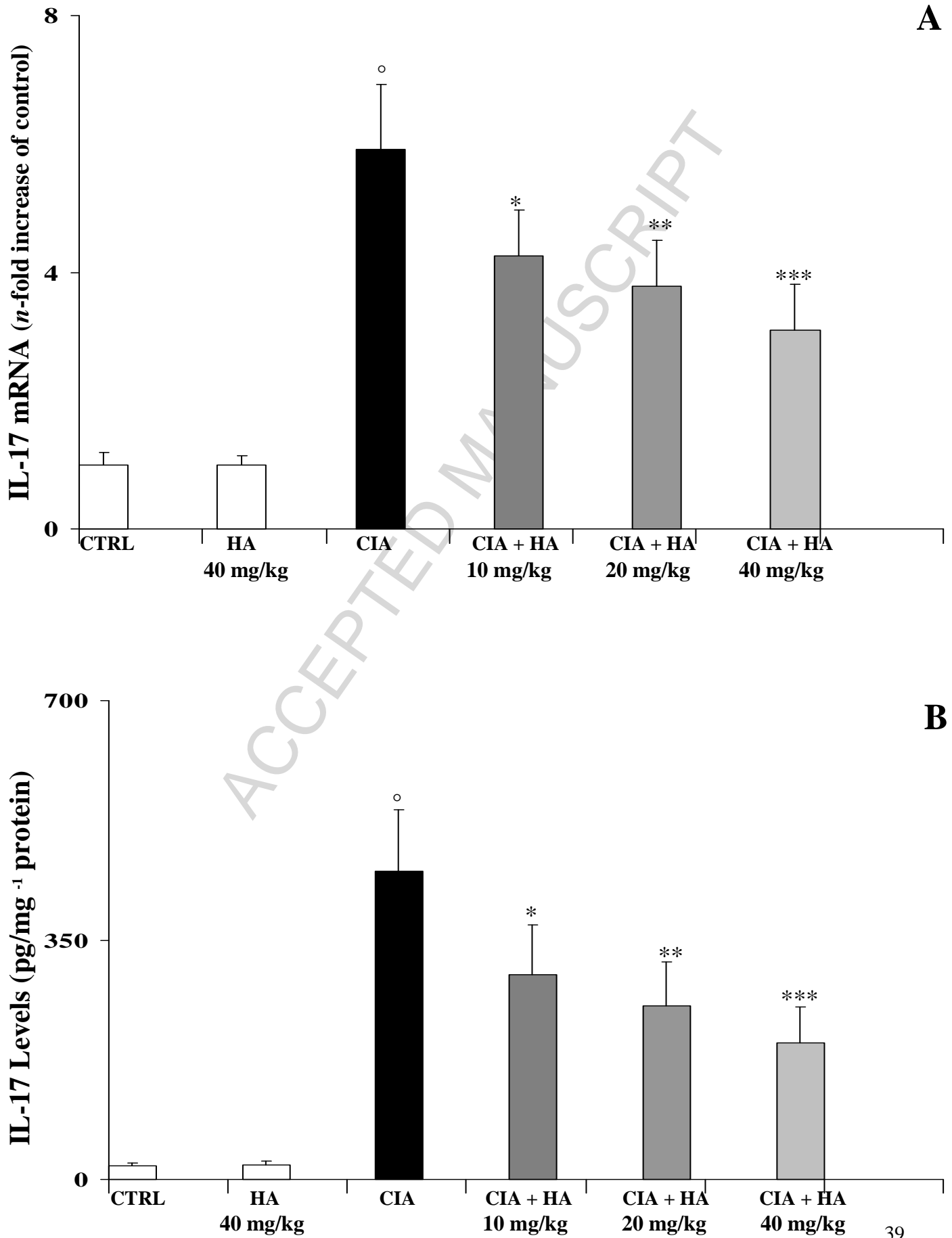


Fig. 9

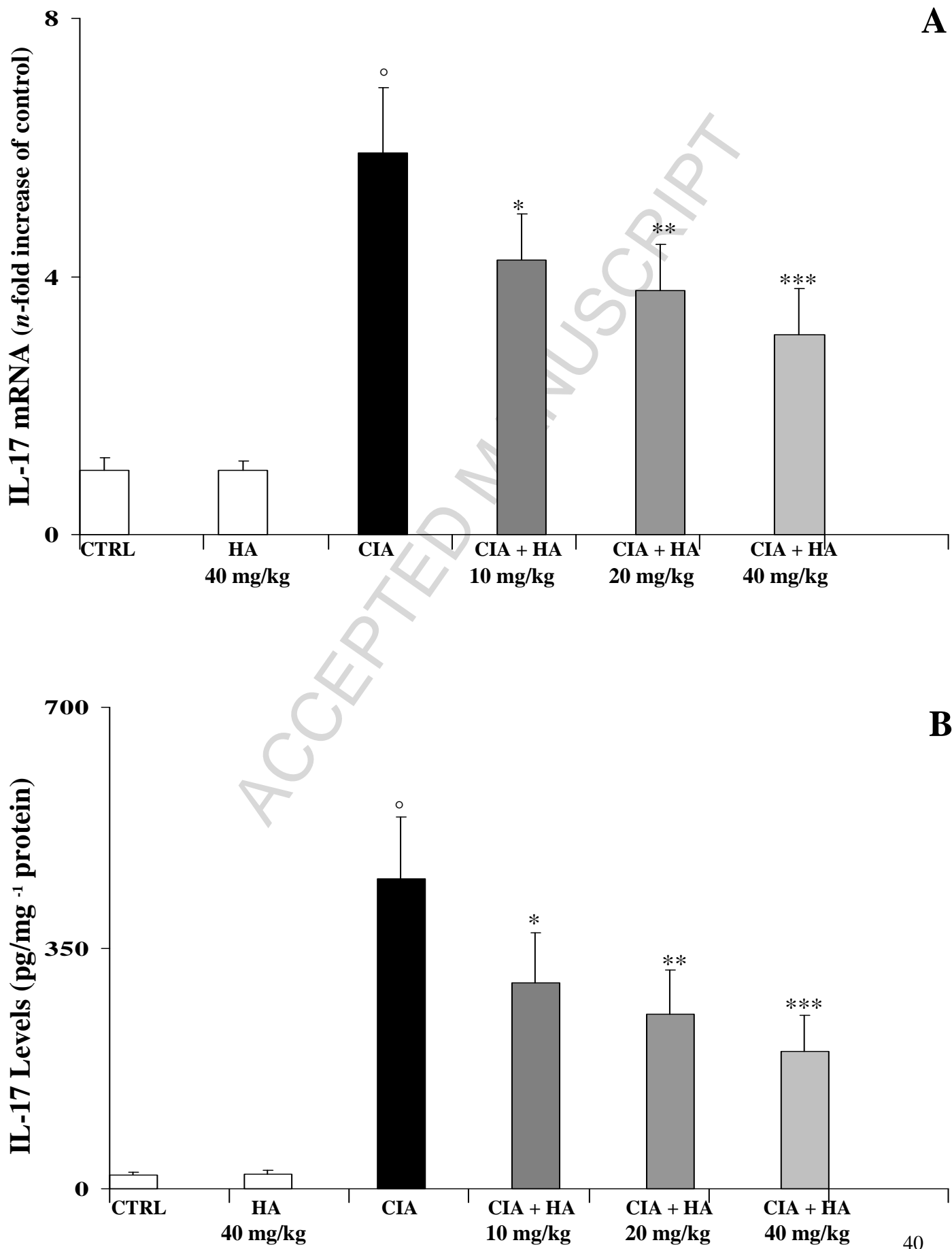


Fig.'32

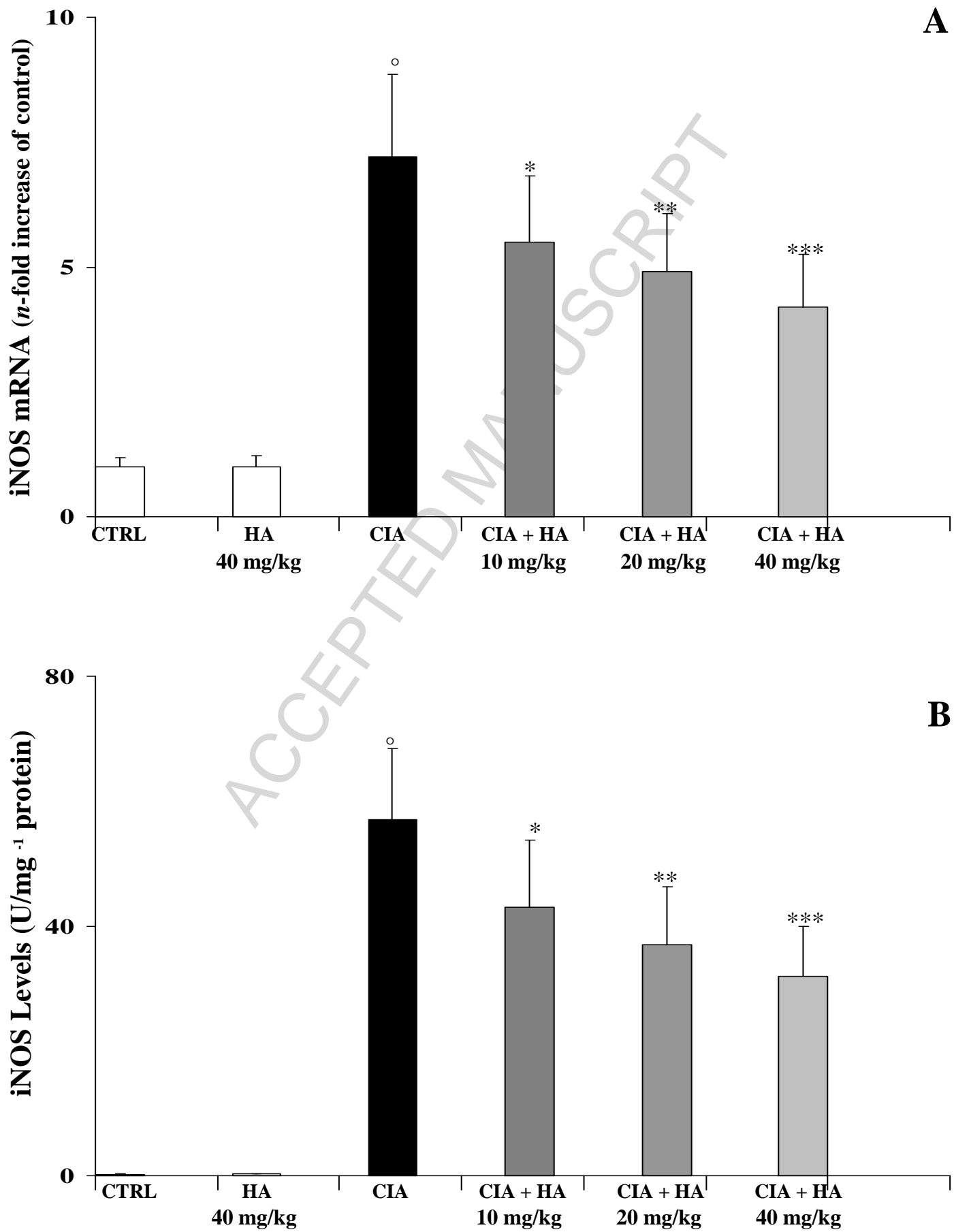
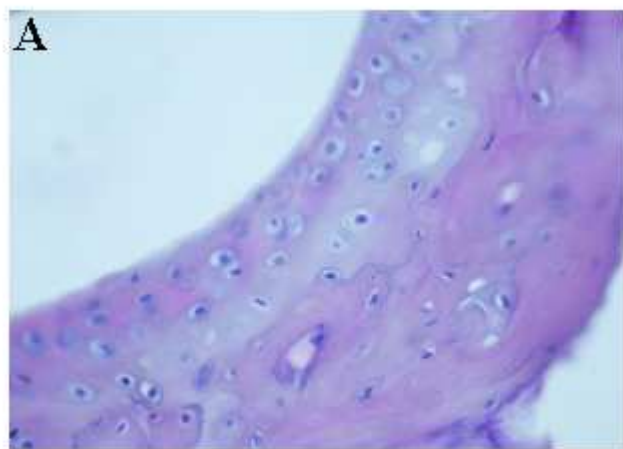
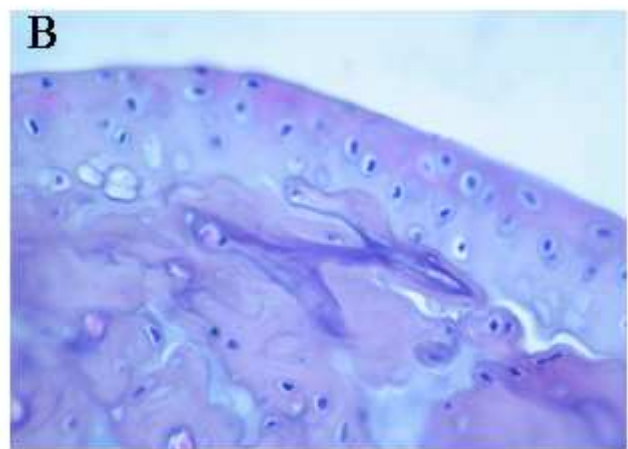


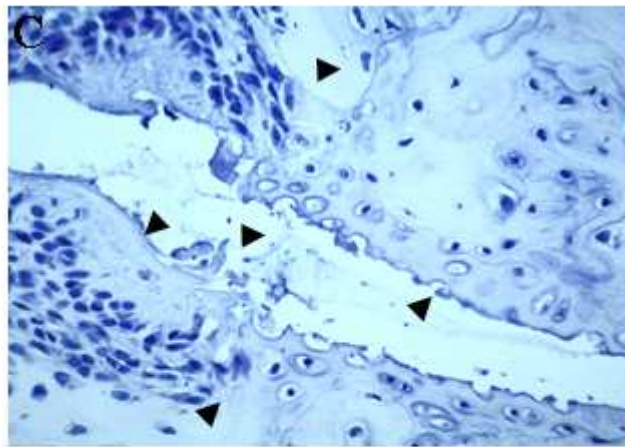
Fig. 11



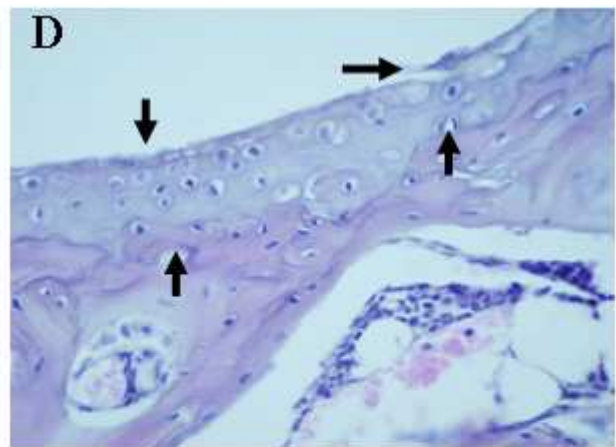
CTRL



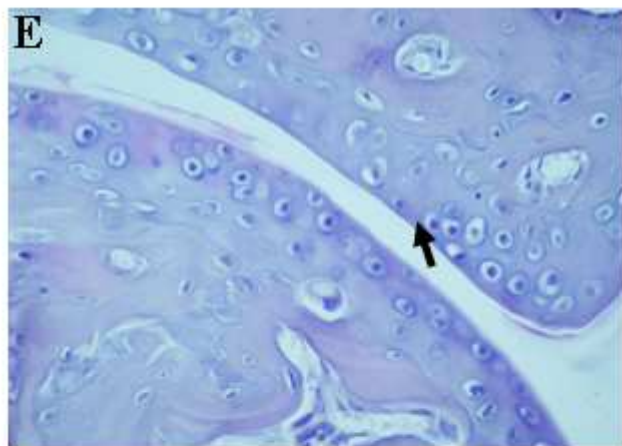
CTRL + HA (40 mg/kg)



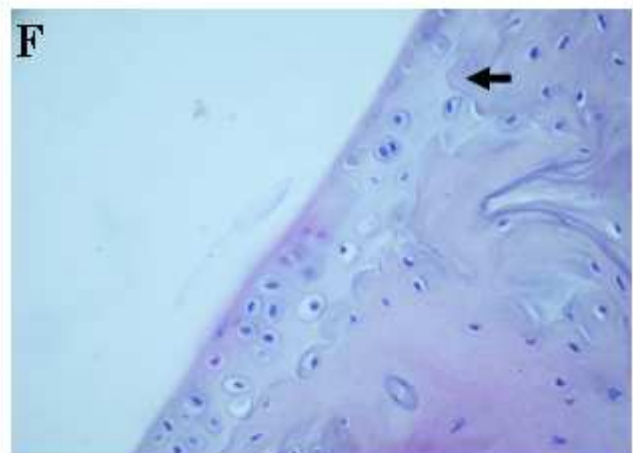
CIA



CIA + HA (10 mg/kg)



CIA + HA (20 mg/kg)



CIA + HA (40 mg/kg)

Fig. 12

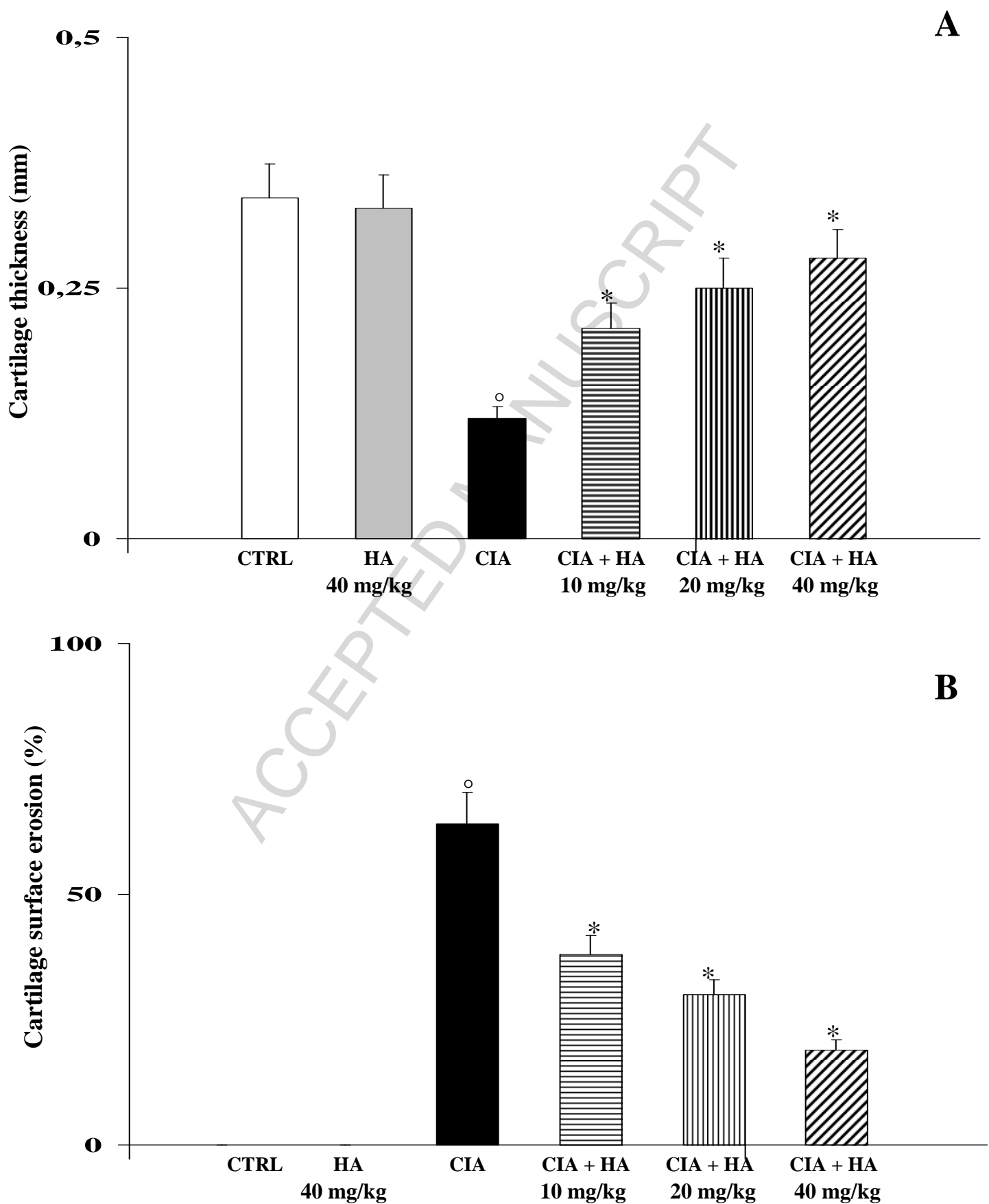


Fig. 13

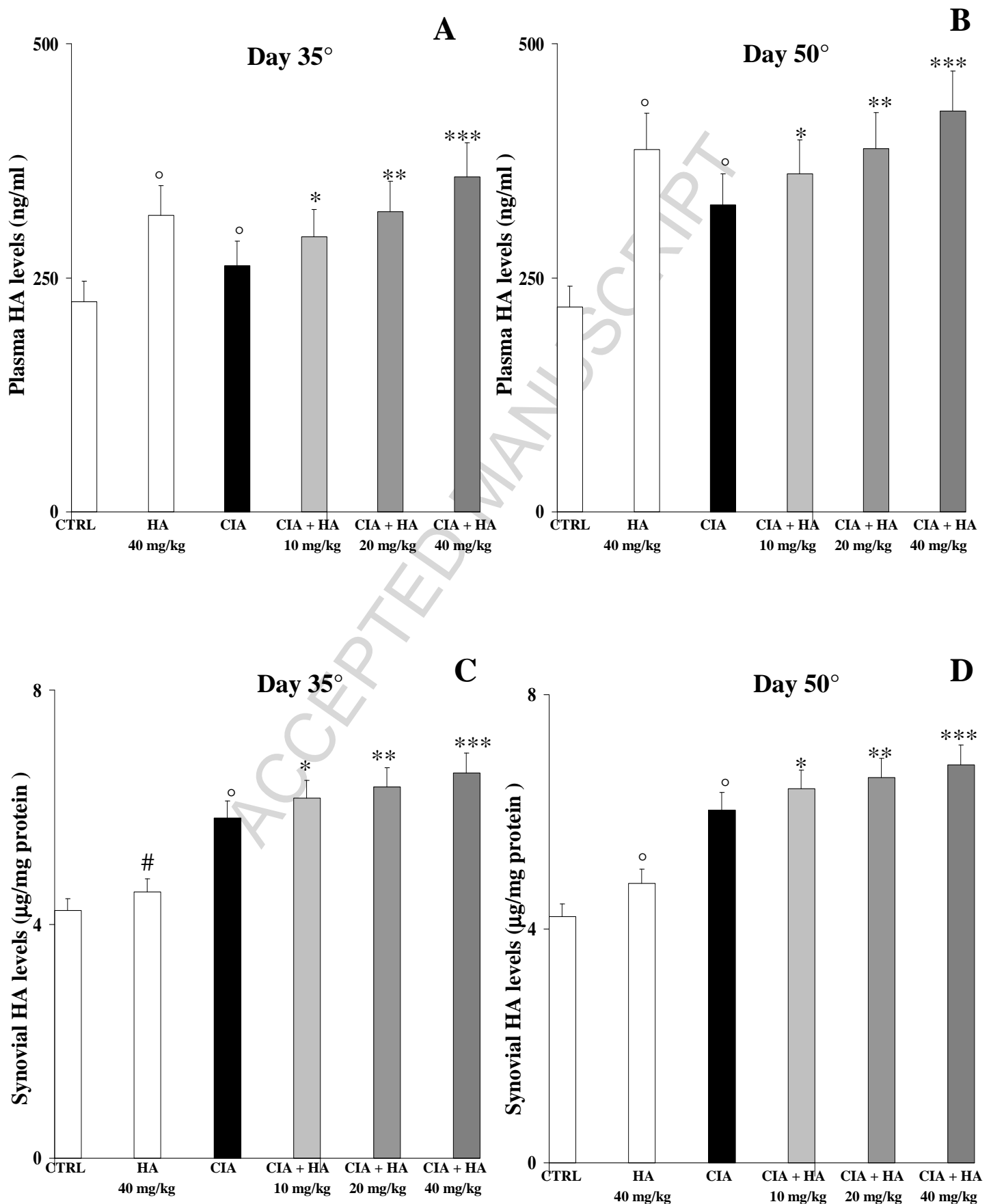


Fig. 14

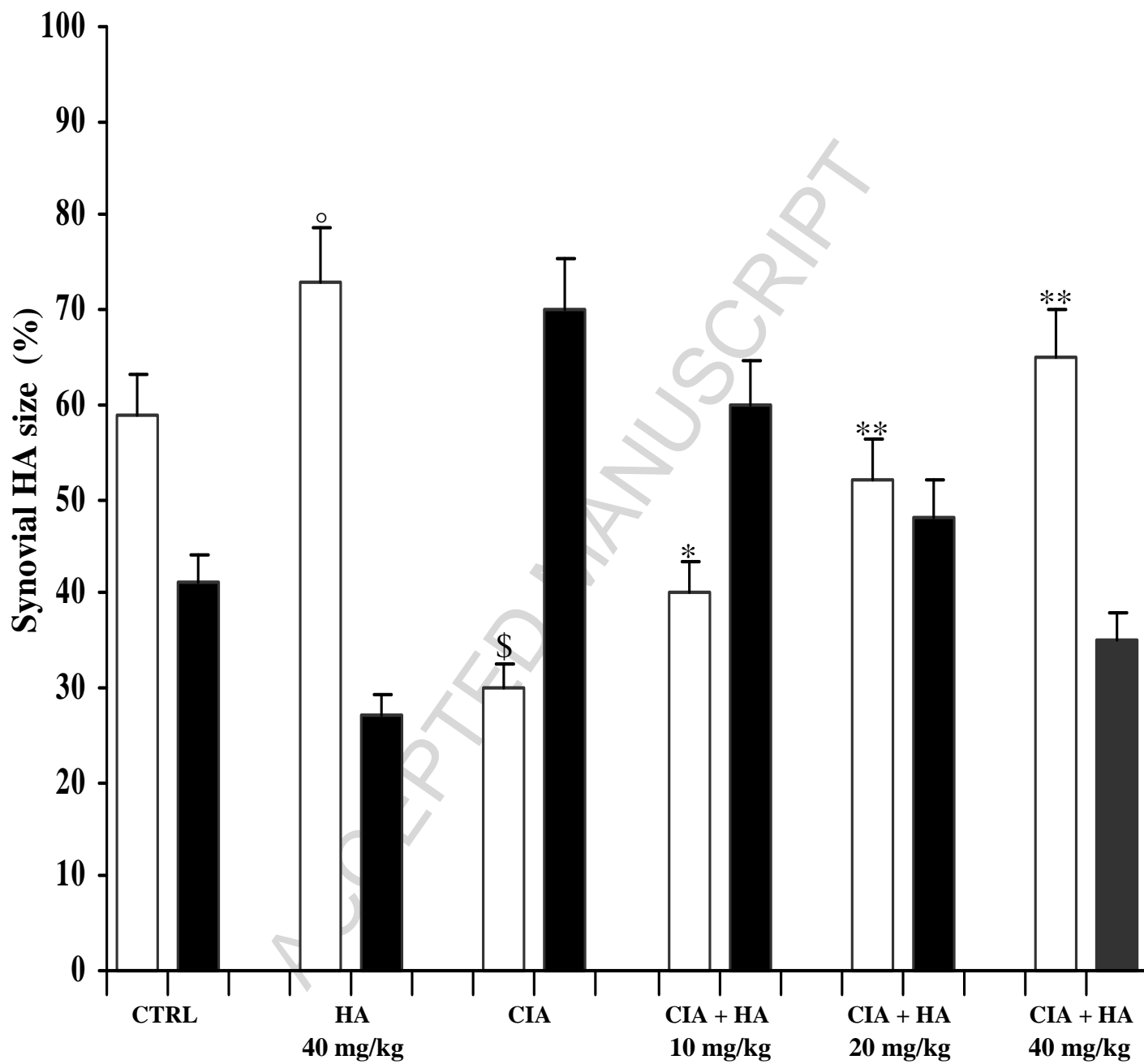


Fig. 15

RESEARCH HIGHLIGHTS

>We investigated the effect of high molecular weight HA on TLR-4 and TLR-2 modulation in CIA mice > Arthritis was induced using bovine type II collagen and Freund's adjuvant. Mice were treated with HA intraperitoneally for 30 days > CIA increased TLR-4, TLR-2, MyD88 and TRAF6 mRNA expression and the related protein in the cartilage of arthritic joints > High levels of TNF- α , IL-1- β , IL-17, MMP-13 and iNOS in the joint of arthritic mice were also detected > HA treatment, at different doses, significantly limited CIA incidence and decreased all the parameters up-regulated by CIA >