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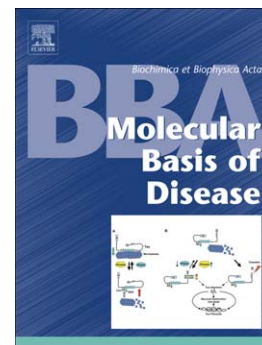
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**HIGH MOLECULAR WEIGHT HYALURONAN REDUCED RENAL PKC ACTIVATION  
IN GENETICALLY DIABETIC MICE**

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**Abstract**

The cluster determinant (CD44) seems to play a key role in tissues injured by diabetes type II. CD44 stimulation activates the protein kinase C (PKC) family which in turn activates the transcriptional nuclear factor kappaB (NF- $\kappa$ B) responsible for the expression of the inflammation mediators such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), interleukin-18 (IL-18), inducible nitric oxide synthase (iNOS) and matrix metalloproteinases (MMPs). Regulation of CD44 interaction with its ligands depends greatly upon PKC. We investigated the effect of the treatment with high molecular weight hyaluronan (HA) on diabetic nephropathy in genetically diabetic mice. BKS.Cg-m+/+Lepr<sup>db</sup> mice had elevated plasma insulin from 15 days of age and high blood sugar levels at four weeks. The severe nephropathy that developed was characterized by a marked increase in CD44 receptors, protein kinase C betaI, betaII and epsilon (PKC $_{\beta$ I, PKC $_{\beta$ II and PKC $_{\epsilon}$ ) mRNA expression and the related protein products in kidney tissue. High levels of mRNA and related protein levels were also detected in the damaged kidney for NF- $\kappa$ B, TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS.

Chronic daily administration of high molecular mass HA for two weeks significantly reduced CD44, PKC $_{\beta$ I, PKC $_{\beta$ II and PKC $_{\alpha}$  gene expression and the related protein production in kidney tissue and TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS expression and levels also decreased. Histological analysis confirmed the biochemical data. However, blood parameters of diabetes were unchanged.

These results suggest that the CD44 and PKC play an important role in diabetes and interaction of high molecular weight HA with these proteins may reduce inflammation and secondary pathologies due to this disease.

## 1. Introduction

Over the last decade, considerable evidence has demonstrated the molecular and cellular links between metabolism and inflammation, particularly in obesity and diabetes. Diabetic nephropathy is one of the major microvascular complications in diabetes and it is the most common single cause of end-stage renal disease worldwide [1]. The development of diabetic nephropathy is described as a process progressing from moderate glomerular hyperfiltration and nephromegaly to frank proteinuria with severe hypertension and marked renal insufficiency until end-stage renal disease [2]. Chronic inflammation, characterized by elevated circulating levels of inflammatory markers, appears to play a critical role in the pathogenesis of type 2 diabetes and its associated complications, particularly diabetic nephropathy and the consequent cardiovascular risk [3]. Recent evidence indicates that chemokines, interleukins, adhesion molecules, growth factors, and free radicals are deeply involved in the development of diabetic nephropathy [4-5]. The cytokines that seem to play a key role in diabetic renal failure include pro-inflammatory cytokines such as tumor necrosis alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-18 (IL-18) [6-8]. Inducible nitric oxide synthase (iNOS), and cytokine-stimulated metalloproteinases (MMPs), especially MMP-7, are also greatly involved in the development of diabetic complications and kidney damage [9-10]. One ROS, nitric oxide (NO), is a highly reactive, cytotoxic molecule that is heavily implicated in diabetes [11]. Increased levels of NO promotes numerous effects on podocytes, including alteration of extracellular matrix synthesis [12], and activation of apoptosis [13].

Biologics that inhibit the signalling cascade mediated by pro-inflammatory cytokines are effective in treating diabetic pathologies by reducing both inflammation and renal failure [6, 14-15]. However, blocking IL-18 and/or TNF- $\alpha$  does not lead to total protection from diabetic complications, indicating that other signalling pathways that mediate inflammation and tissue injury have still to be elucidated [6, 14-15].

Hyaluronan (HA) is a linear polymer composed of repeating disaccharides of *D*-glucuronic acid - *N*-acetyl *D*-glucosamine [16]. HA can exist as a high-molecular-weight polymer ( $10^6$  D) or in lower

molecular weight forms. Low molecular mass HA in particular is produced after tissue injury [17]. Several findings have shown that HA functions are determined in part by the size of the molecule. High molecular weight HA is suggested to play a structural role and to promote tissue integrity, while low molecular weight HA may be a signal of tissue injury [18-20].

The best-known HA receptor is CD44 [21], this is a transmembrane glycoprotein which is widely found in T lymphocytes, granulocytes, monocytes, fibroblasts, keratinocytes, and epithelial cells. CD44 stimulation with HA plays a role in various physiological functions, such as cell-adhesion, cell-substrate interactions, and lymphocyte recruitment, as well as in pathological processes such as chronic inflammation and metastasis of malignant cells [22]. A role for CD44, as HA receptor, in the regulation of inflammation *in vivo* has been shown by studies in which anti-CD44 treatment reduces not only inflammation in experimental diabetes [23-24], but also leukocyte migration in skin-associated immune disease [25].

A number of reports have shown PKC members to be greatly involved in the mediation of inflammation [26]. The regulation of CD44 interaction with its ligands depends heavily on PKC which modulates the phosphorylation state of CD44 [27] and the various anchoring proteins [28]. Interestingly, PKC activation leads to the redistribution of CD44 receptors in membrane ruffles located at the leading edge of glioma cells [29]; a key feature of inflammation and invading cells. PKC therefore has the potential to modulate both the affinity of CD44 for HA [30] as well as the localization of the receptor over the cell surface [29]. It has also been reported that the activation of the CD44 receptor by HA produces PKC activation [31], thus establishing a concatenated mechanism between the CD44 receptor and PKC. In addition, these processes are to a large extent mediated by the increased expression of pro-inflammatory cytokines and other detrimental molecules such as NO and MMPs, which are in turn controlled by the activation of key transcriptional nuclear factor kB (NF-kB) [32].

As previous investigations reported that the inhibition of PKC isoforms such as PKC $_{\alpha}$ , PKC $_{\beta I}$  and PKC $_{\beta II}$  reduces diabetic complications, especially diabetic nephropathy [33-35], and since we

demonstrated in a previous study that high molecular mass HA is able to reduce phorbol-12-myristate-13-acetate (PMA)-induced inflammation in mouse chondrocytes by interacting with PKC [19], the aim of this study was to investigate whether high molecular weight HA administration, at different concentrations, influences CD44 and PKC function in congenic diabetic BKS.Cg-m<sup>+/+</sup>Lepr<sup>db</sup> mice.

## 2. Materials and Methods

### 2.1. Animals.

Male congenic diabetic BKS.Cg-m<sup>+/+</sup>Lepr<sup>db</sup> mice 15 weeks old with a mean weight of 25-30 g were used in our study. Mice, purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) 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=12); 2) Control + HA (30 mg/kg) (n=12); 3) Diabetic (n=17); 4) Diabetic + HA (7.5 mg/kg) (n=17); 5) Diabetic + HA (15 mg/kg) (n=17); 6) Diabetic + HA (30 mg/kg) (n=17).

### 2.2. Materials.

High molecular weight HA (4,000,000 Da) was purchased from Sigma-Aldrich Srl, (Milan, Italy). Mouse TNF- $\alpha$  (cat. IB49688), IL-6 (cat. IB49686), IL-18 (cat. IB49697) commercial ELISA kits were provided by Immuno-Biological laboratories Inc. (Minneapolis, MN, USA). Mouse MMP-7 (cat. E0102Mu) and iNOS (cat. E0837Mu) commercial ELISA kits were obtained from USCN Life Science Inc. Wuhan (Wuhan, China). Mouse CD44 monoclonal antibodies were supplied by Millipore (Billerica, MA, USA), mouse PKC $\alpha$  monoclonal antibodies were supplied by Abcam plc (Cambridge, UK), mouse PKC $\beta$ II polyclonal antibodies were supplied by Sigma-Aldrich (Milan, Italy) and PKC $\beta$ I polyclonal antibodies and Horseradish peroxidase-labeled goat anti-rabbit

antibodies 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. HA treatment.

HA was dissolved in saline solution (0.9% NaCl) at three different concentrations (7.5, 15 and 30 mg/kg and administered intraperitoneally at a volume of 1.0 ml/kg body weight, once a day starting from day one up until day 15.

### 2.4. Plasma glucose and insulin

Glucose was evaluated in plasma samples (100  $\mu$ l) at the end of the experiment. Concentrations were assayed using commercial clinical test kits (cat.GAGO20) obtained from Sigma-Aldrich (Milan, Italy). Insulin levels were also measured in plasma samples (10  $\mu$ l) at the end of the experiment using an ELISA commercial kit (cat. EZRMI-13K) provided by Millipore (Billerica, MA, USA).

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

Total RNA was isolated from renal tissue for reverse-PCR real time analysis of CD44, PKC $\beta$ <sub>I</sub>, PKC $\beta$ <sub>II</sub>, PKC $\alpha$ , TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS (RealTime PCR system, Mod. 7500, Applied Biosystems, USA) using an Omnizol Reagent Kit (Euroclone, West York, UK). The first strand of cDNA was synthesized from 1.0  $\mu$ g total RNA using a high capacity cDNA Archive kit (Applied Biosystems, USA).  $\beta$ -actin mRNA was used as an endogenous control to allow the relative quantification of. CD44, PKC $\beta$ <sub>I</sub>, PKC $\beta$ <sub>II</sub>, PKC $\alpha$ , TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS. PCR RealTime was performed by means of ready-to-use assays (Assays on demand, Applied Biosystems) on both targets and endogenous controls. The amplified PCR products were quantified by measuring the calculated cycle thresholds ( $C_T$ ) of CD44, PKC $\beta$ <sub>I</sub>, PKC $\beta$ <sub>II</sub>, PKC $\alpha$ , TNF- $\alpha$ , IL-6, IL-18, MMP-7, iNOS, and  $\beta$ -Actin mRNA. The amounts of specific mRNA in samples were calculated using 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).

#### 2.6. Western blot assay of CD44, PKC $\beta_I$ , PKC $\beta_{II}$ , and PKC $\alpha$ proteins

For SDS-PAGE and Western blotting, the kidney tissue 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  $\mu$ l/well) were separated on a mini gel (10%). The proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences) using a semi-dry apparatus (Bio-Rad). 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.

#### 2.7. NF- $\kappa$ B p50/65 transcription factor assay

NF- $\kappa$ B p50/65 DNA binding activity in nuclear extracts of kidney tissue samples was evaluated in order to measure the degree of NF- $\kappa$ B activation. Analysis was performed in line with the manufacturer's protocol for a commercial kit (NF- $\kappa$ B p50/65 Transcription Factor Assay Colorimetric, cat. n°SGT510, Chemicon International, USA). In brief, one of the kidney was

removed from the animals at the end of the experimental phase, washed in ice-cold 10 mM Tris-HCl, pH 7.4, and blotted on absorbent paper. Samples were then plotted, using an automatic plotter, in order to isolate renal cells. Cytosolic and nuclear extraction was performed by lysing the cell membrane with an appropriate hypotonic lysis buffer containing protease inhibitor cocktail and tributylphosphine (TBP) as reducing agent. After centrifugation at 8,000 x g, the supernatant containing the cytosolic fraction was discarded, while the pellet containing the nuclear portion was then re-suspended in the apposite extraction buffer and the nuclei were disrupted by a series of drawing and ejecting actions. The nuclear suspension was then centrifuged at 16,000 x g. The supernatant fraction was the nuclear extract. After the determination of protein concentration and adjustment to a final concentration of approximately 4.0 mg/ml, this extract was stored in aliquots at -80° C for the subsequent NF-kB 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  $\lambda$  450 nm. Values are expressed as relative optical density (OD) per mg protein.

#### 2.8. *TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS ELISA assay*

Tissue samples obtained from mice kidneys in the presence of 1.0 nM PMSF and protease inhibitor cocktail were first lysed using a suitable hypotonic lysis buffer and then centrifuged at 13,000 rpm for 10 min at 4°C. The analysis of TNF- $\alpha$ , IL-6, and IL-18 was carried out using a specific commercial kit. Briefly, 50  $\mu$ l of standards, samples and controls were added to each well of the coated microplate. 50  $\mu$ l of each specific biotin-conjugate antibody was then added to each well. After 120 min of incubation at 20-22°C, the liquid from the wells was discarded, the wells were washed three times and 100  $\mu$ l of Streptavidin-HRP was added. After further incubation for 60 min and having washed the wells once more, 100  $\mu$ l of a substrate chromogen solution was added. After 10 min incubation and the addition of 100  $\mu$ l of stop solution, the absorbance of each well was read

spectrophotometrically at  $\lambda$  450 nm. TNF- $\alpha$  IL-6 and IL-18 values are expressed as pg/mg protein. For MMP-7 and iNOS, 100  $\mu$ 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 from each well was discarded. After adding 100  $\mu$ 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 ml 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 aspired then wells were washed with wash buffer. 90  $\mu$ l of the substrate solution was added and after incubation at room temperature for 30 minutes, 50  $\mu$ l of stop solution was added. Within 30 minutes, the absorbance of each well was read spectrophotometrically at  $\lambda$  450 nm. MMP-7 values are expressed as pg/mg protein, while iNOS values are expressed as U/mg protein.

### 2.9. Histology

At the end of the experimental phase, the right kidney was first excised and then perfused with 4% paraformaldehyde (PFA)/0.1 M phosphate buffer (PB), decapsulated and immersed in 4% PFA/0.1 M PB. Then kidneys were dehydrated in graded ethanol and embedded in a special tissue embedding media (Paraplast, McCormick Scientific, Richmond, VA, USA). Sections of 4  $\mu$ m thickness were cut with a microtome (mod. RM 2125 RT, Leica Camera AG, Solms, Germany), stained with Hematoxylin-Eosin technique and viewed and photographed with a light microscope (mod. BH2, Olympus America Inc., Melville, USA).

### 2.10. 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 [36].

### 2.11. 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  $p < 0.05$ .

### 2.12. Statement of animal care

The studies reported in this manuscript were carried 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 plasma glucose and insulin

At the age of four weeks the acute expression of the db/db mutation commonly induces noticeable alterations in body phenotype, tissue indexes, and the endocrine-related parameters of BKS.Cg-m<sup>+/+</sup>Lepr<sup>db</sup> mice relative to controls [37]. Increased body weight in these mice is related to concomitant elevations in blood glucose and insulin levels when compared with control values [37]. The 15-day HA treatment period, at all doses, had no effect on plasma glucose and insulin in both diabetic and control mice (data not shown).

### 3.2. CD44, PKC $\beta$ I, PKC $\beta$ II and PKC $\alpha$ mRNA expression and Western blot analysis.

CD44, PKC $\beta$ I, PKC $\beta$ II and PKC $\alpha$  (Figs. 1, 2, 3 and 4) mRNA evaluation (panels A of each Figure) and Western blot analysis with densitometric evaluation (Figs. 1, 2, 3 and 4, panels B and C of each Figure) were assayed in order to estimate the degree of CD44 activation and the consequent cell signalling pathway booster that culminates in NF- $\kappa$ B factor activation. Very low gene expression and the related protein production of CD44, PKC $\beta$ I, PKC $\beta$ II and PKC $\alpha$  were obtained in non-diabetic

mice (CTRL and CTRL+HA). In contrast, a marked increase in the expression and protein synthesis of the CD44 receptor and their signal mediators PKC $\beta$ I, PKC $\beta$ II and PKC $\alpha$  were found in the kidney tissue of diabetic mice. This means that CD44 receptor and the PKC pathway are involved in the inflammatory mechanism consequent to this pathology.

The treatment with high molecular weight HA for 15 days significantly reduced CD44 receptor activation and its signal mediator levels in diabetic mice. As reported in Figs. 1, 2, 3 and 4, HA was able to lower CD44, PKC $\beta$ I, PKC $\beta$ II and PKC $\alpha$  expression and protein synthesis at all concentrations in a dose-dependent manner.

### 3.3. *NF-kB activation.*

Fig. 5 shows the changes in the NF-kB p50/p65 heterodimer translocation over the course of the experiment. NF-kB DNA binding was present at very low levels in the renal tissue of non-diabetic mice. In contrast, diabetic complications induced massive NF-kB translocation into the nucleus of the damaged podocytes; the treatment of diabetic mice with HA at different concentrations reduced NF-kB activation in a dose-dependent manner, thereby confirming the effect on CD44, PKC $\beta$ I, PKC $\beta$ II and PKC $\alpha$  parameters.

### 3.4. *TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS mRNA expression and ELISA assay.*

TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS were assayed because of their active role in inflammation and diabetic nephropathy. (Figs. 6, 7, 8, 9 and 10). Their mRNA evaluation (panel A of each Figure) and protein concentration (panel B of each Figure) showed that the mRNA of these detrimental mediators was not stimulated in non-diabetic mice and protein level was very low. However, in diabetic mice, TNF- $\alpha$ , IL-6, IL-18, MMP-7 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. 6, 7, 8, 9, and 10; panels A and B of each Figure), the increase in mRNA expression of TNF- $\alpha$ , IL-6, IL-18,

MMP-7 and iNOS in diabetic 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*

Glomerular injury in the diabetic mice can be early characterized by glomerular hyperplasia and mesangial matrix expansion. Fig. 11 demonstrates a representative kidney histopathology of all the experimental groups. Panels A and B show a normal kidney tissue section from undiabetic control mice untreated or treated with HA. In contrast, as reported in Figure 11 panel C, the glomerular hyperplasia and mainly the mesangial cell increment is clear evident. In panels D, E, and F a representative selection of kidney tissue sections from diabetic mice receiving HA treatment shows that a gradual reduction in glomerular hyperplasia and mesangial matrix expansion occurred. As indicated by the intensive coloration the number of mesangial cells increased dramatically.

## 4. Discussion

Diabetes is a chronic disease in which patients develop dramatic and devastating complications. The most severe complication of diabetes is nephropathy, which frequently causes death in diabetic patients and accounts for a large number of cases of end-stage renal disease.

In the present study, we investigated the effects of high molecular weight HA, at different concentrations, on CD44 receptor modulation in congenitally diabetic mice. This study suggests that highly polymerized HA may reduce diabetes-induced renal injury by modulating CD44 signalling, in contrast with low molecular weight HA which may exert the opposite effects as previously demonstrated [17-20]. HA effects were dose dependent and were able to reduce not only the expression of CD44 receptor and of PKC $\beta$ I, PKC $\beta$ II, and PKC $\alpha$  but also NF-kB activation and the increment of inflammatory cytokines, such as IL-6 and IL-18, and of MMP-7 and iNOS that

were up-regulated in diabetic mice. Biochemical results were further confirmed by the reduction in glomerulosclerosis as revealed by the histological analysis.

HA possesses an extraordinarily rapid half-life. It is quickly eliminated by means of CD44 receptors on liver endothelium. At a normal plasma concentration the half-life is approximately 4-6 minutes, while at higher plasma concentrations the HA half-life is considerably prolonged to 18-43 minutes [38]. The intraperitoneal HA half-life is unknown, but is probably also very rapid, and also in this case an increase in the HA dose administered may result in a prolonged HA half-life with a consequent major bioavailability to different organs. Therefore we might expect a linear progression of effect by increasing the HA range concentrations administered. However, due to its viscous properties, it is really difficult to administer concentrations higher than 30 mg/kg.

The interaction between cell surface CD44 and extracellular HA are implicated in a variety of physiological and pathological processes, such as the uptake and degradation of HA, T cell activation, leukocyte extravasation at inflammation sites, and tumor metastasis [39]. CD44 expression in the renal tissue correlates with the degree of the nephropathy in diabetic rats [40], and at high levels in several other form of nephropathy stimulates the increment in inflammatory mediators that are the main cause of renal damage [41-42].

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases that consists of a number of isoforms [43]. The differences between the PKC isoforms dependent on their substrate specificity suggest that a particular PKC isoform within a single cell may regulate specific physiological responses. PKCs regulate a variety of biological functions including ion channel activity, cell differentiation, and other biochemical and physiological responses to various stimuli [44-45]. The PKC/PKD family exerts numerous effects on cellular pathways that are important in inflammation and has previously been considered as a potential therapeutic target in diabetes [33-35, 46]. PKC is able to modify the phosphorylation state of the CD44 receptor and the interaction with HA [27], and PKC in turn may be activated through different pathways, among these by CD44 stimulation [31]. Therefore, CD44 and PKC are interdependent and greatly involved in the

inflammation cascade. NF- $\kappa$ B promotes the transcription of several genes including cytokines and other inflammation products [32]. CD44 stimulation activates members of the PKC family, such as PKC $\alpha$  and PKC $\beta$  [47–48], which in turn activate the NF- $\kappa$ B responsible for the expression of inflammation mediators such as TNF- $\alpha$ , IL-6, IL-18, iNOS, its dangerous free radical product, NO, and MMPs responsible for diabetic nephropathy [5-13, 49-51].

Cell interaction with the surrounding extracellular matrix is fundamental in many physiological and pathological mechanisms. Proteoglycans (PGs) may influence cell behaviour through binding events mediated by their glycosaminoglycan (GAG) chains. The binding affinity of the interaction depends on the ability of the oligosaccharide sequence to provide an optimal charge and surface with the protein [52]. The interaction of HA degradation products with CD44 provides signals to initiate inflammation [22-23, 25]. Other data support the idea that a balance between low molecular weight HA and high molecular weight HA may control the activation of inflammation [53]. In fact, these preliminary findings suggest that high molecular weight HA is inactive for CD44 modulation, while low molecular weight HA originating from depolymerised HA at the inflammation site is active. [17-18, 54]. We previously reported that high molecular weight HA is able to reduce inflammation stimulated by PMA in mouse chondrocytes. By using a specific CD44 blocking antibody it was demonstrated that PKC and not CD44 receptor is the target for HA action [19]. We suggest the same mechanism to explain the findings of the present study, in which chronic daily administration of high molecular mass HA for 2 weeks significantly reduced CD44, PKC $\beta$ I, PKC $\beta$ II and PKC $\alpha$  gene expression and the related protein production in kidney tissue and TNF- $\alpha$ , IL-6, IL-18, MMP-7 and iNOS expression levels.

The effect exerted by HA on the parameters considered could be due to the ability of HA to bind protein structures, such as PKC, thereby exerting a block that reduced NF- $\kappa$ B activation. A similar effect exerted directly on the CD44 receptor should be excluded, since the high molecular mass HA interaction with CD44 seems to be just one step in a pathway of intermediary metabolism involving the catabolism of native HA into small fragments [55]. The rationale for the use of high molecular



mass HA in place of a specific antibody targeted at CD44 may be explained by the fact that a block of the CD44 receptor leads to the abrogation of several fundamental CD44 functions, such as cell adhesion, cell-substrate interaction, and lymphocyte recruitment [39].

Blocking PKC isoforms using specific agents was also previously attempted but the effects are not very encouraging because of the high toxicity of these agents [56]. The choice of HA as modulating agent derived essentially from its natural endogenous origin. The number of active charged HA sites (carboxylic groups) is responsible for HA action. In fact, high molecular weight HA contains many charged groups that may completely wrap protein structures such as PKC, thereby preventing its activation. The mechanism could be similar to a well known lock-and-key model which applies to all the better-known effector-receptors where the effector is chemically specific and a similar molecule with several charged groups may block the receptor. In this work, the larger size of HA molecule was able to mask the PKC active site completely. The resultant inhibition of PKC thereby produced a reduction in NF- $\kappa$ B activation that in turn limited the transcription of the detrimental inflammatory intermediates with a consequent reduction in tissue injury, in particular in the limitation of diabetic nephropathy as the results of this study reported.

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

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**Figure legends***Fig. 1*

Effect of HA treatment on CD44 mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. 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 CD44 protein levels.  $^{\circ}p<0.001$  vs Control;  $*p<0.01$ ,  $**p<0.005$  and  $***p<0.001$  vs diabetic.

*Fig. 2*

Effect of HA treatment on PKC $_{\beta I}$  mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. 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 PKC $_{\beta I}$  protein levels.  $^{\circ}p<0.001$  vs Control;  $*p<0.01$ ,  $**p<0.005$  and  $***p<0.001$  vs diabetic.

*Fig. 3*

Effect of HA treatment on PKC $_{\beta II}$  mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. 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 PKC $_{\beta II}$  protein levels.  $^{\circ}p<0.001$  vs Control;  $*p<0.01$ ,  $**p<0.005$  and  $***p<0.001$  vs diabetic.

*Fig. 4*

Effect of HA treatment on PKC $_{\alpha}$  mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. 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 PKC $\alpha$  protein levels.  $^{\circ}p<0.001$  vs Control;  $*p<0.01$ ,  $**p<0.005$  and  $***p<0.001$  vs diabetic.

*Fig. 5*

Effect of HA treatment on NF- $\kappa$ B p50/65 transcription factor DNA binding activity in kidney tissue of diabetic mice. 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  $\lambda$  450 nm/mg protein of nuclear extract.  $^{\circ}p<0.001$  vs Control;  $*p<0.005$  and  $**p<0.001$  vs diabetic.

*Fig. 6*

Effect of HA treatment on TNF- $\alpha$  mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. 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- $\alpha$  protein levels.  $^{\circ}p<0.001$  vs Control;  $*p<0.01$ ,  $**p<0.005$  and  $***p<0.001$  vs diabetic.

*Fig. 7*

Effect of HA treatment on IL-6 mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. 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 IL-6 protein levels.  $^{\circ}p<0.001$  vs Control;  $*p<0.01$ ,  $**p<0.005$  and  $***p<0.001$  vs diabetic.

*Fig. 8*

Effect of HA treatment on IL-18 mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. 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 IL-18 protein levels. °p<0.001 vs Control; \*p<0.01, \*\*p<0.005 and \*\*\*p<0.001 vs diabetic.

*Fig. 9*

Effect of HA treatment on MMP-7 mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. Values are the mean ± 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 MMP-7 protein levels. °p<0.001 vs Control; \*p<0.05, \*\*p<0.01 and \*\*\*p<0.005 vs diabetic.

*Fig. 10*

Effect of HA treatment on iNOS mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. Values are the mean ± 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 iNOS protein levels. °p<0.001 vs Control; \*p<0.05, \*\*p<0.01 and \*\*\*p<0.005 vs diabetic.

*Fig.11*

Effect of HA on the prevention of glomerular hyperplasia and mesangial matrix expansion in congenic diabetic mice. Hematoxylin-Eosin staining. Bar = 50 µm. ( magnification 2080 X).

This study could be an additional step to for a better understanding of the complex mechanism that underlies diabetes. Of particular interest is the possibility to obtain useful data for the future development of new molecules that are active in the prevention and the treatment of diabetes.

The research in this field has made significant progress in the last years. Many studies have redefined the molecular basis of diabetes. The conventional view, although fundamental, has been supplanted by a new vision, one clearly supported by experimental and clinical data, of the diabetes disease, especially for type 2 diabetes which is age-related. In fact, it is now clear that inflammation is a key process in the onset of diabetes, and that the inflammatory milieu in this detrimental disease significantly contributes to the development of many of the complications of diabetes, including diabetic nephropathy. The links between this inflammatory state and the development and progression of diabetic nephropathy involve a highly complex network of processes. Diverse inflammatory molecules play significant roles in this scenario, including CD44, PKC isoforms, chemoattractant cytokines, pro-inflammatory cytokines, ROS and MMPs. Consequently, on the basis of this new knowledge, a totally new approach can be planned in order to modulate the inflammatory mechanism that seems to underlie diabetic disease. It is widely recognised that, diabetic nephropathy is one of the worst consequences of this disease. At present, the current treatments available for diabetes complications are still sub-optimal, both in terms of preventing their appearance as well as in halting their progression. A better understanding of the role of inflammatory molecules and processes in the context of diabetes complications will facilitate the development of new and improved therapeutic targets and strategies that can be translated successfully into clinical applications.

It is under this new vision of the molecular basis of diabetic complications that we have performed our research study, aiming to clarify some important aspects of this disease and its damaging consequences that are currently not investigated or not well clarified. The obtained results confirmed the central role played by the CD44 receptor and the activation of PKC isoforms as well as the damage exerted by pro-inflammatory cytokines and the other inflammation mediators. The protective role exerted by high molecular weight hyaluronan could be useful for future pharmacological strategies development in the diabetic complication treatment .

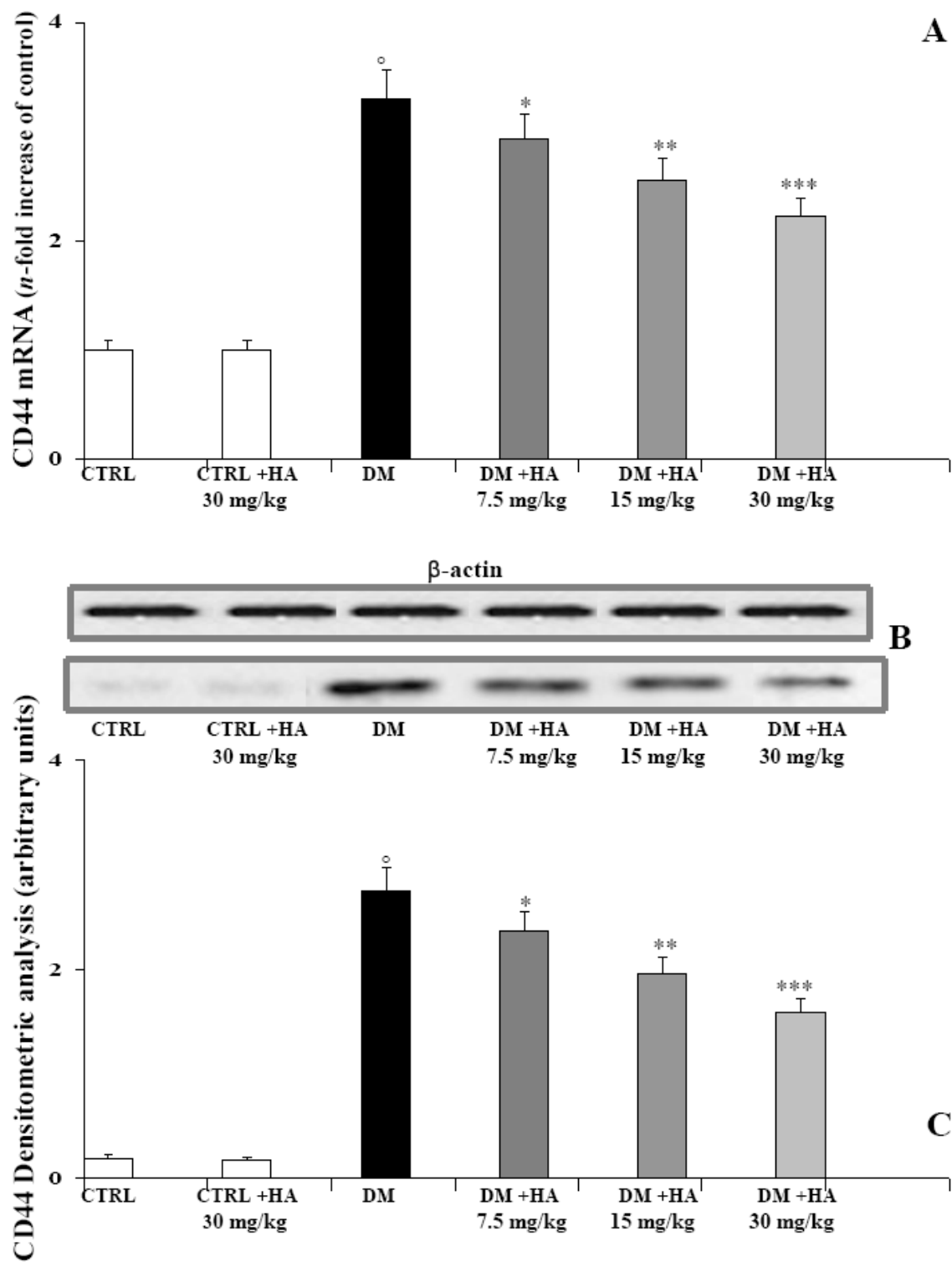


Fig. 1

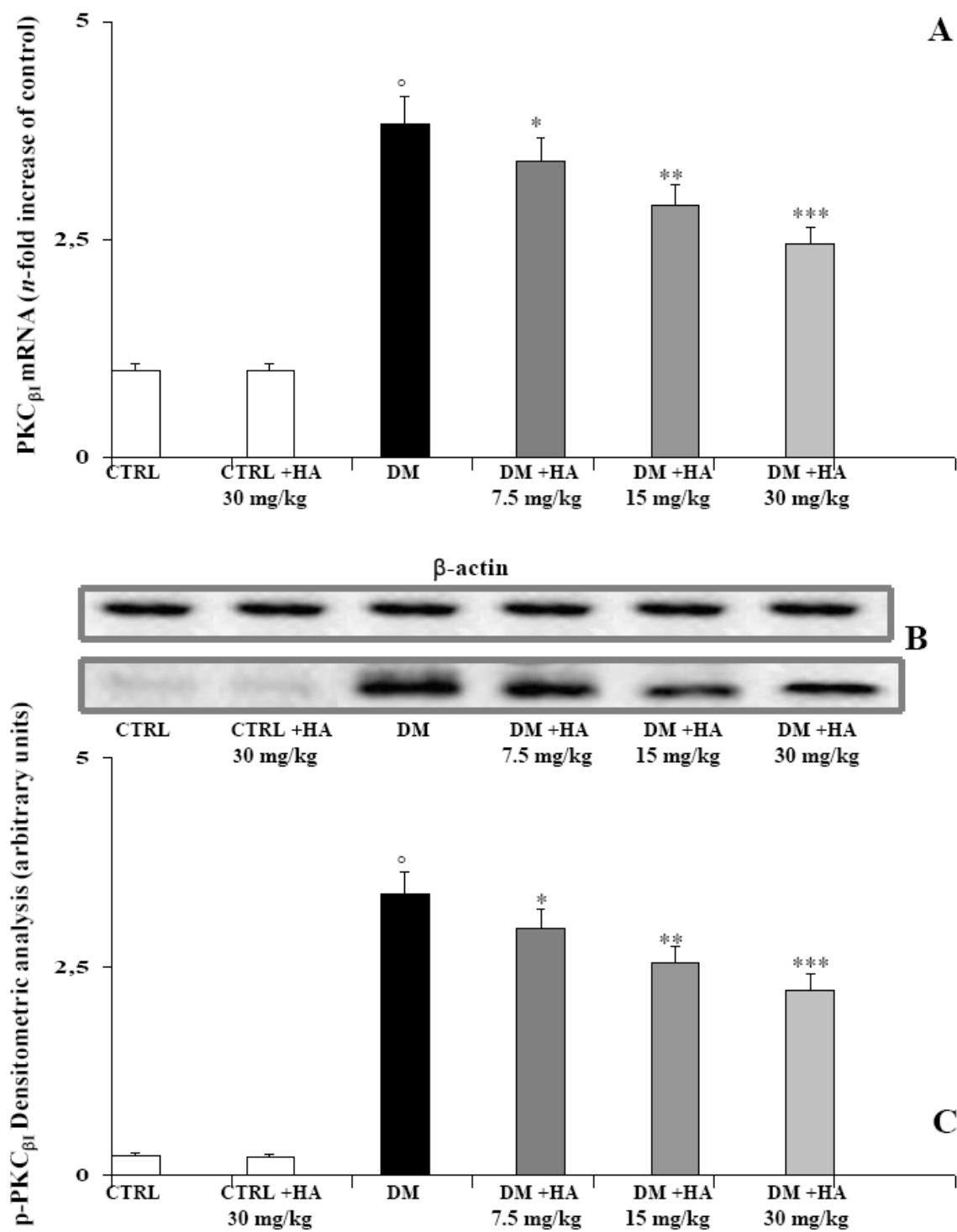


Fig. 2

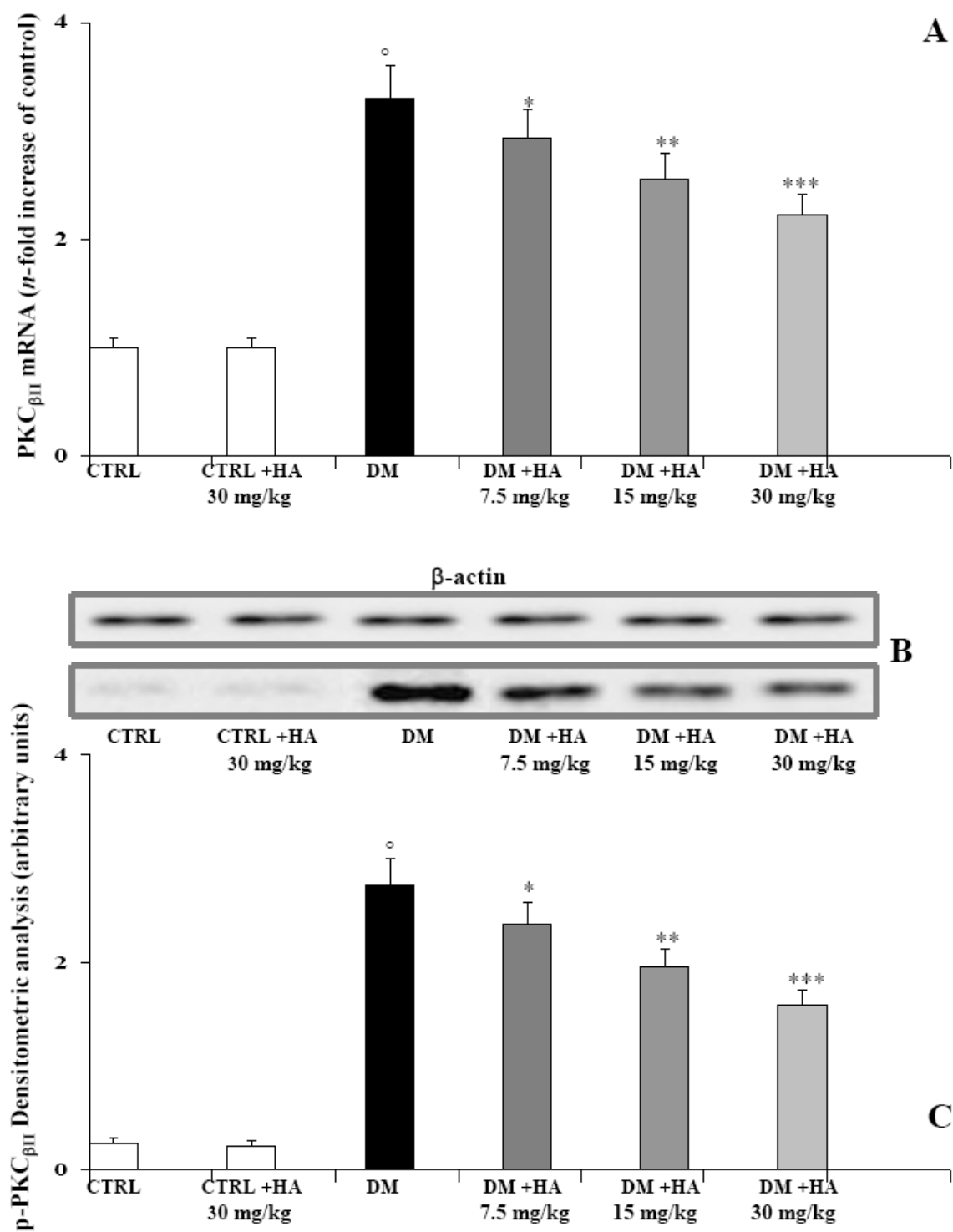


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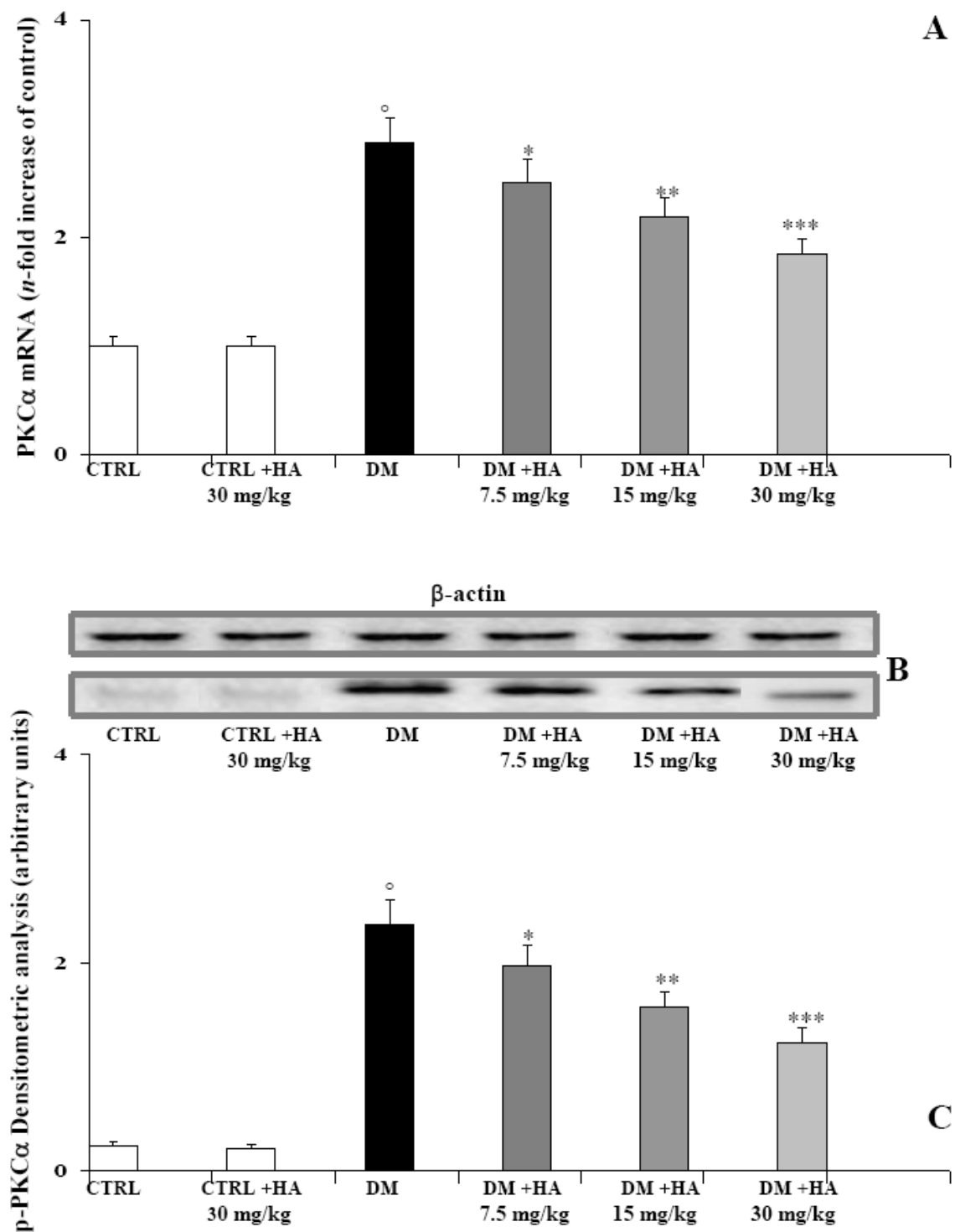


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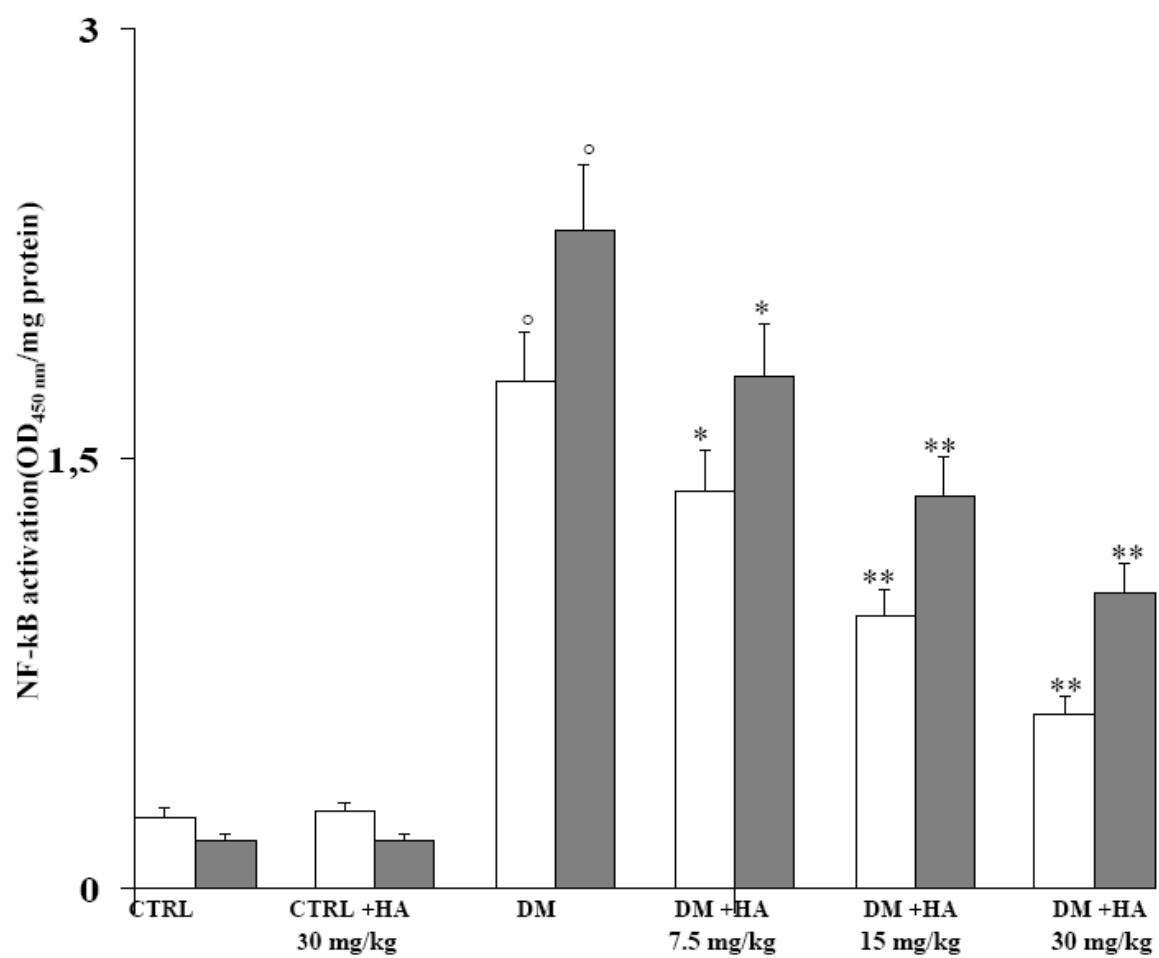


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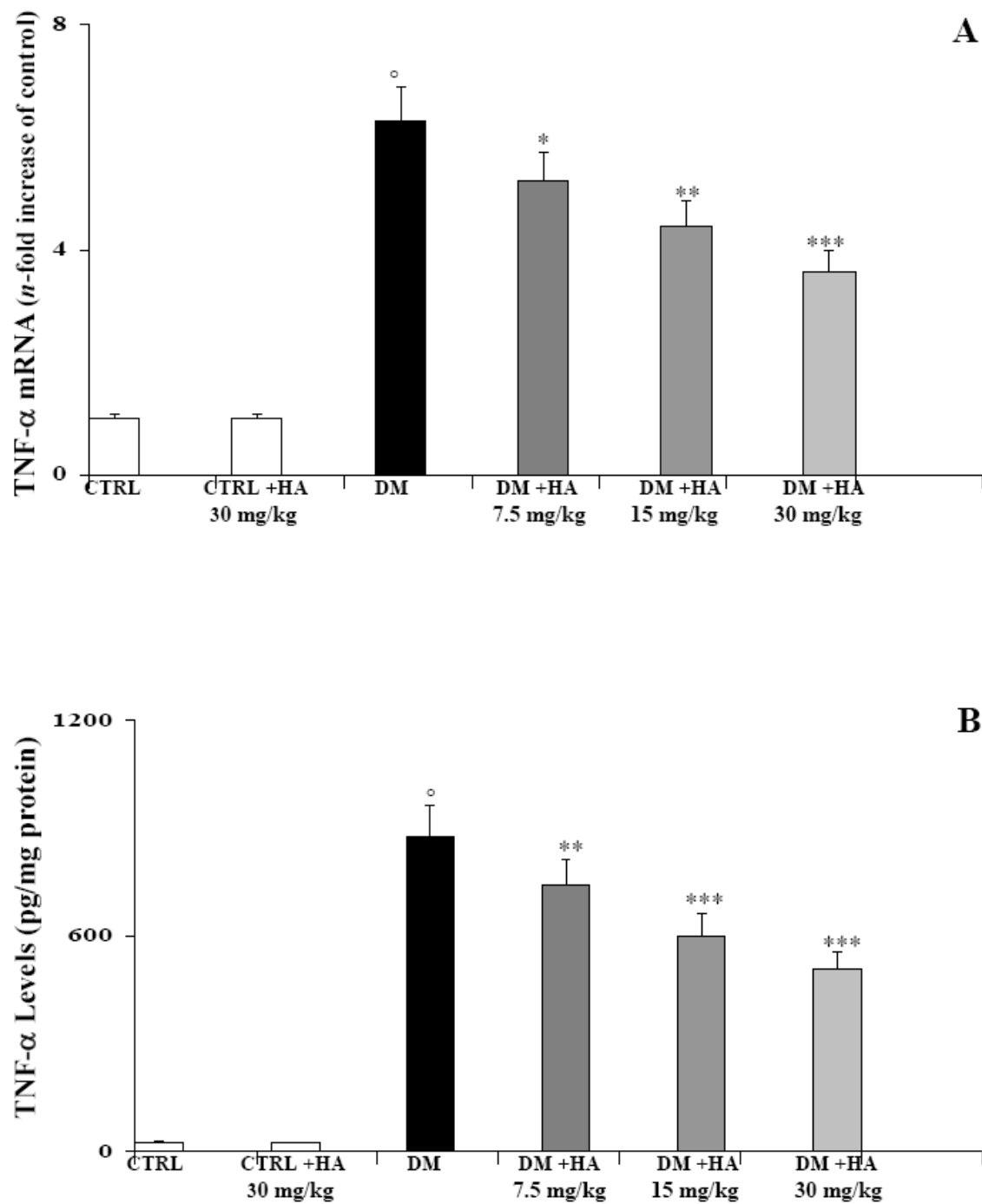


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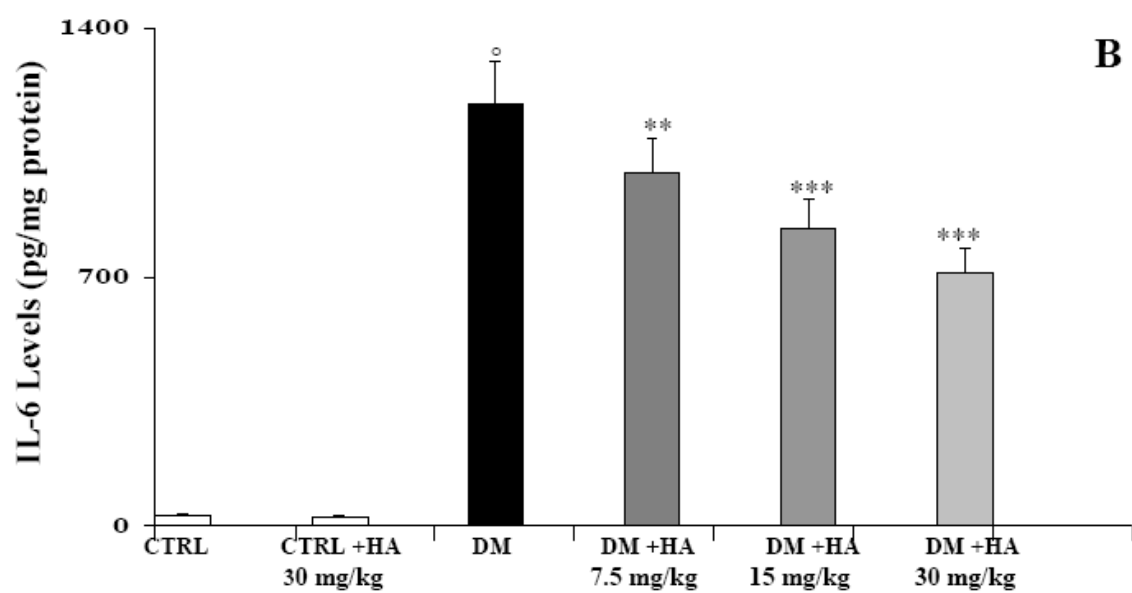
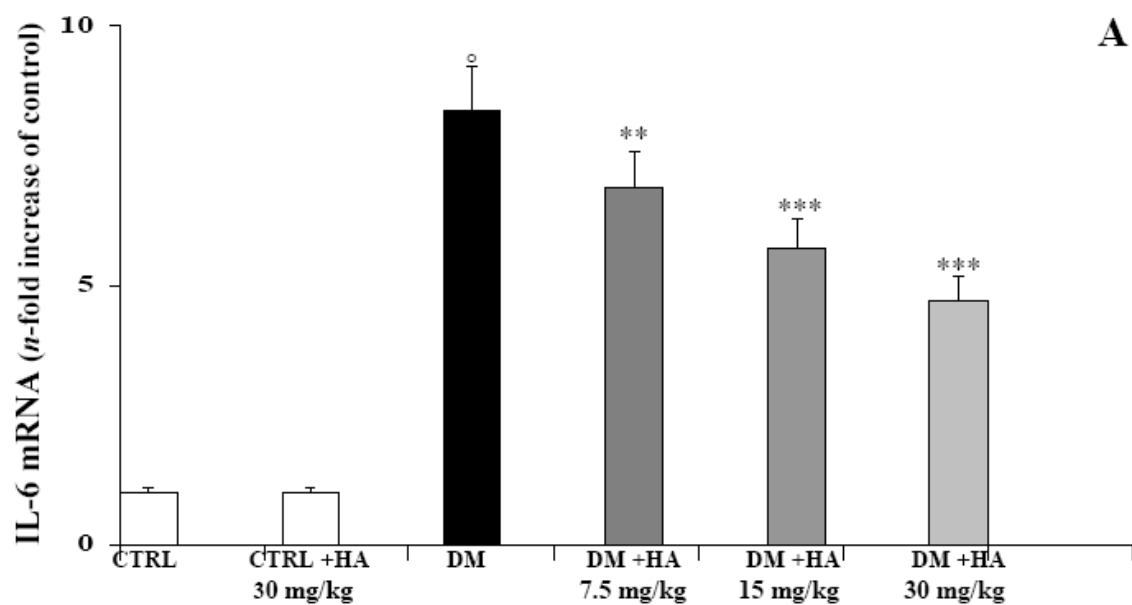


Fig. 7

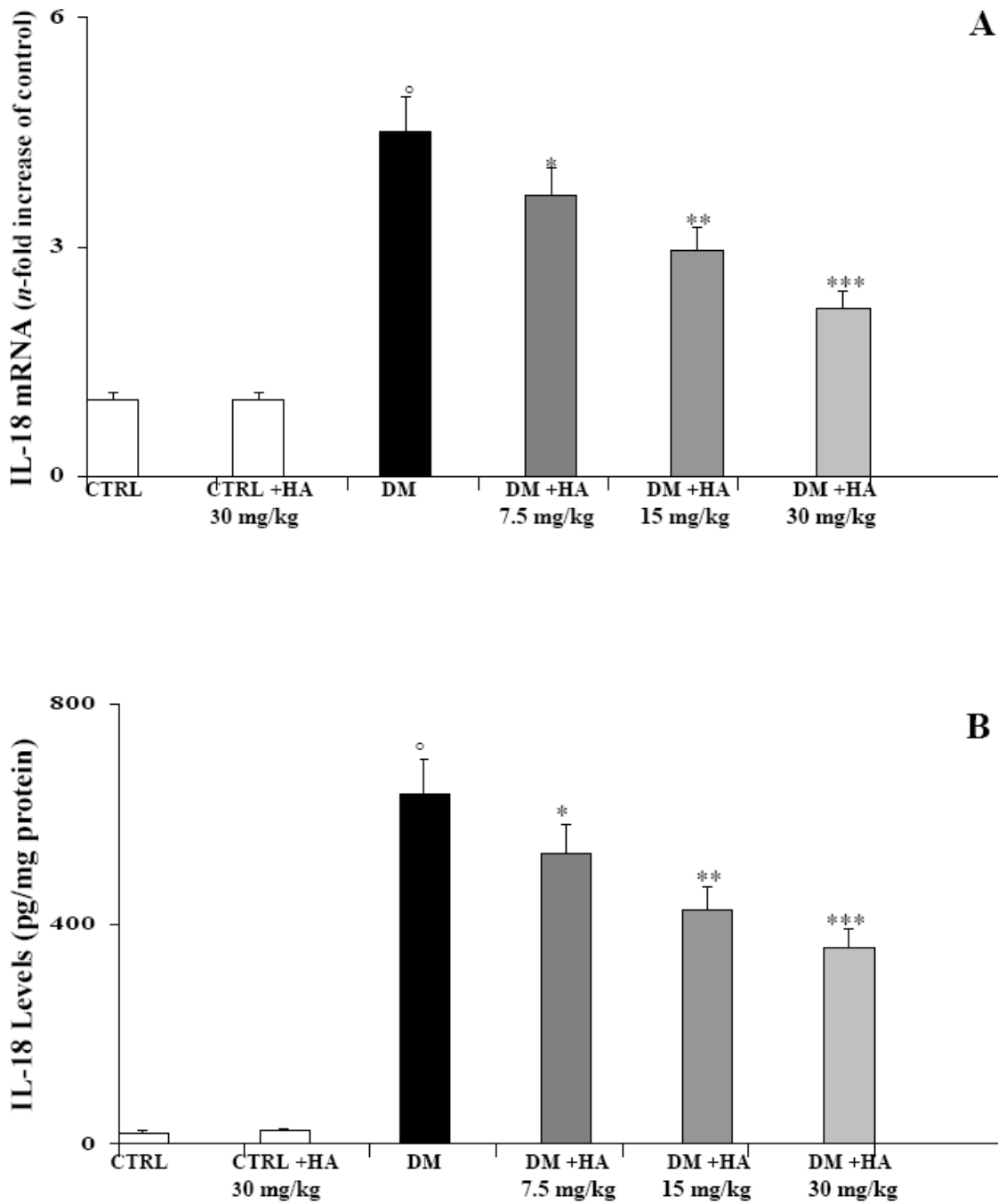


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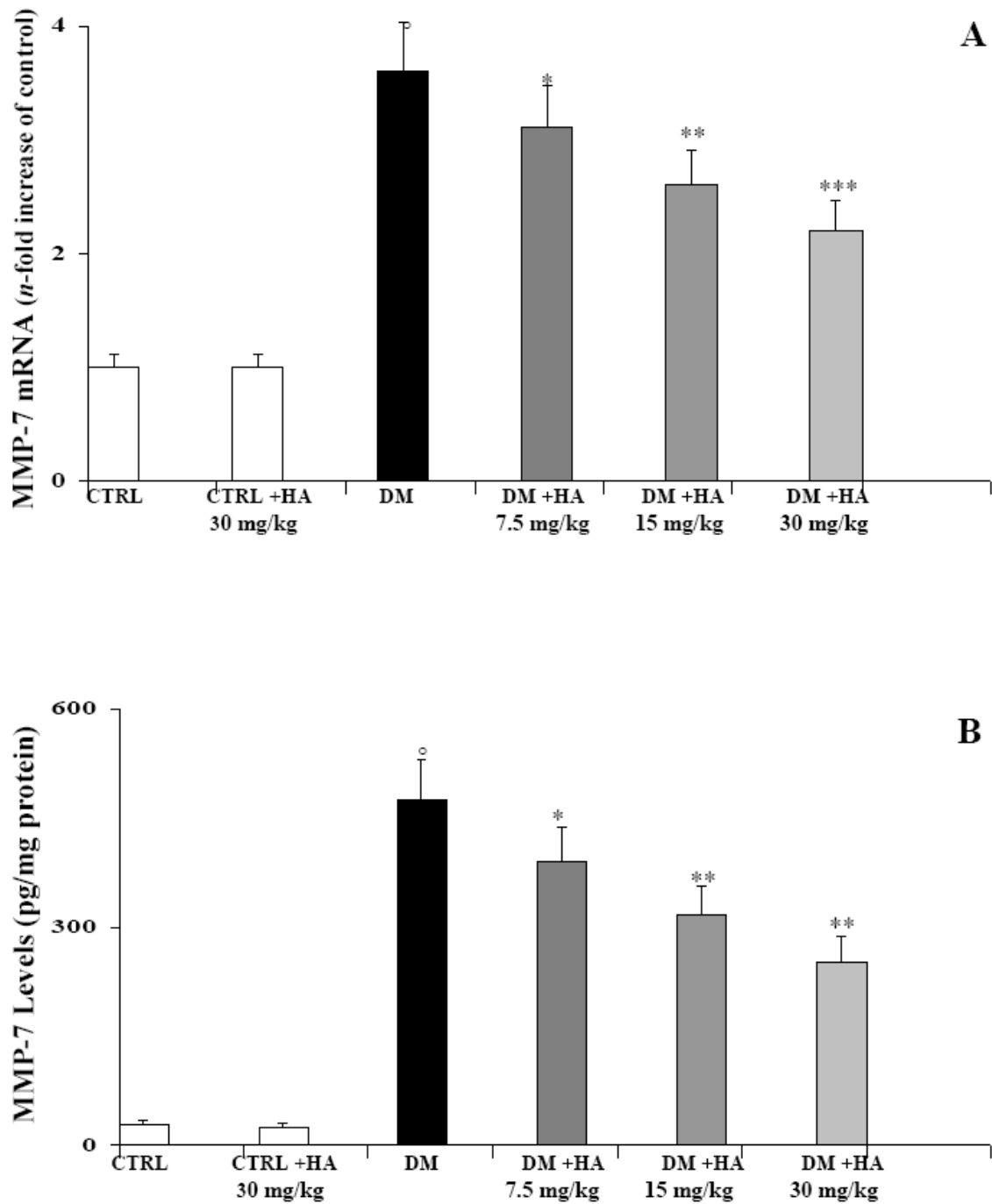


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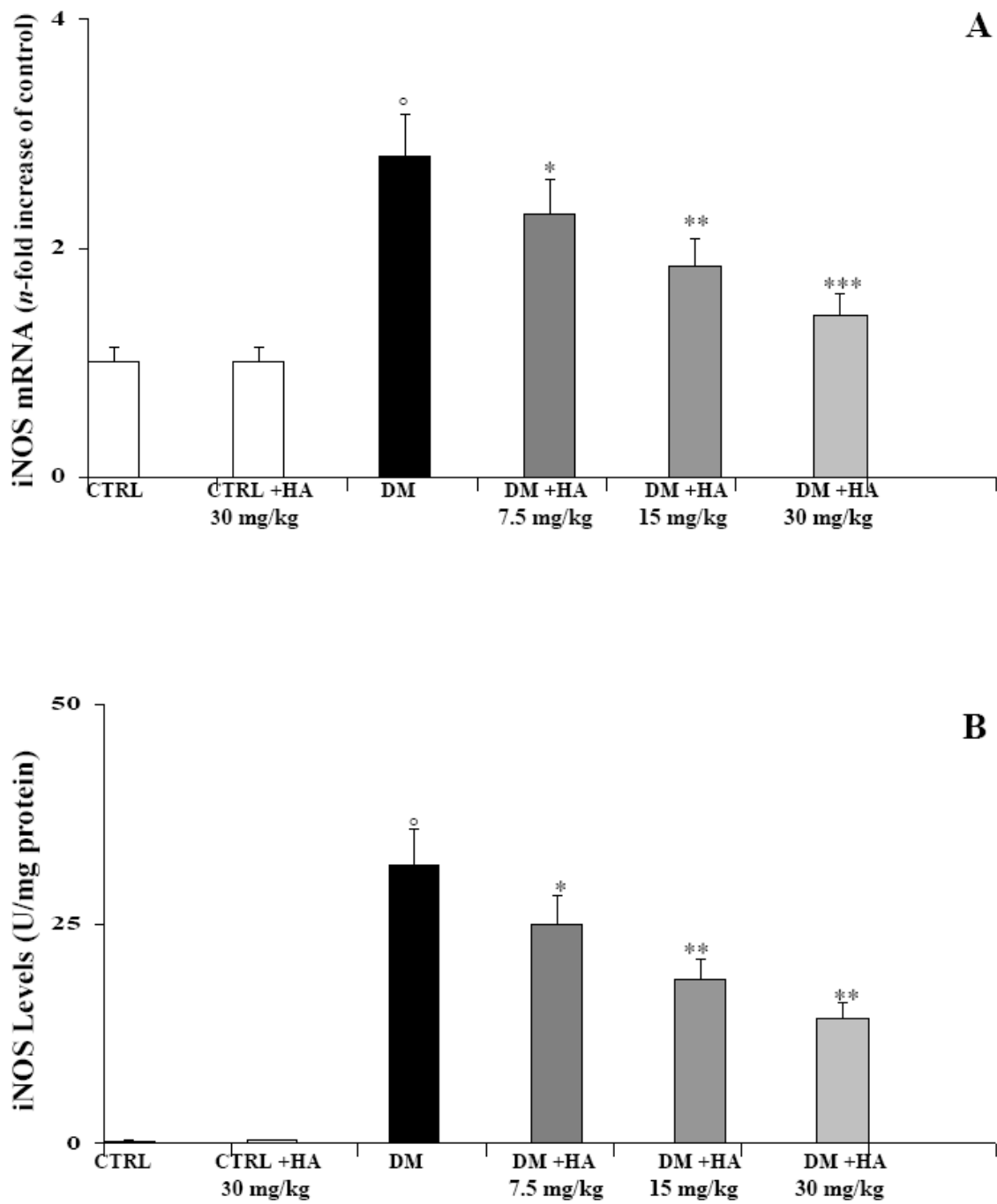


Fig. 10

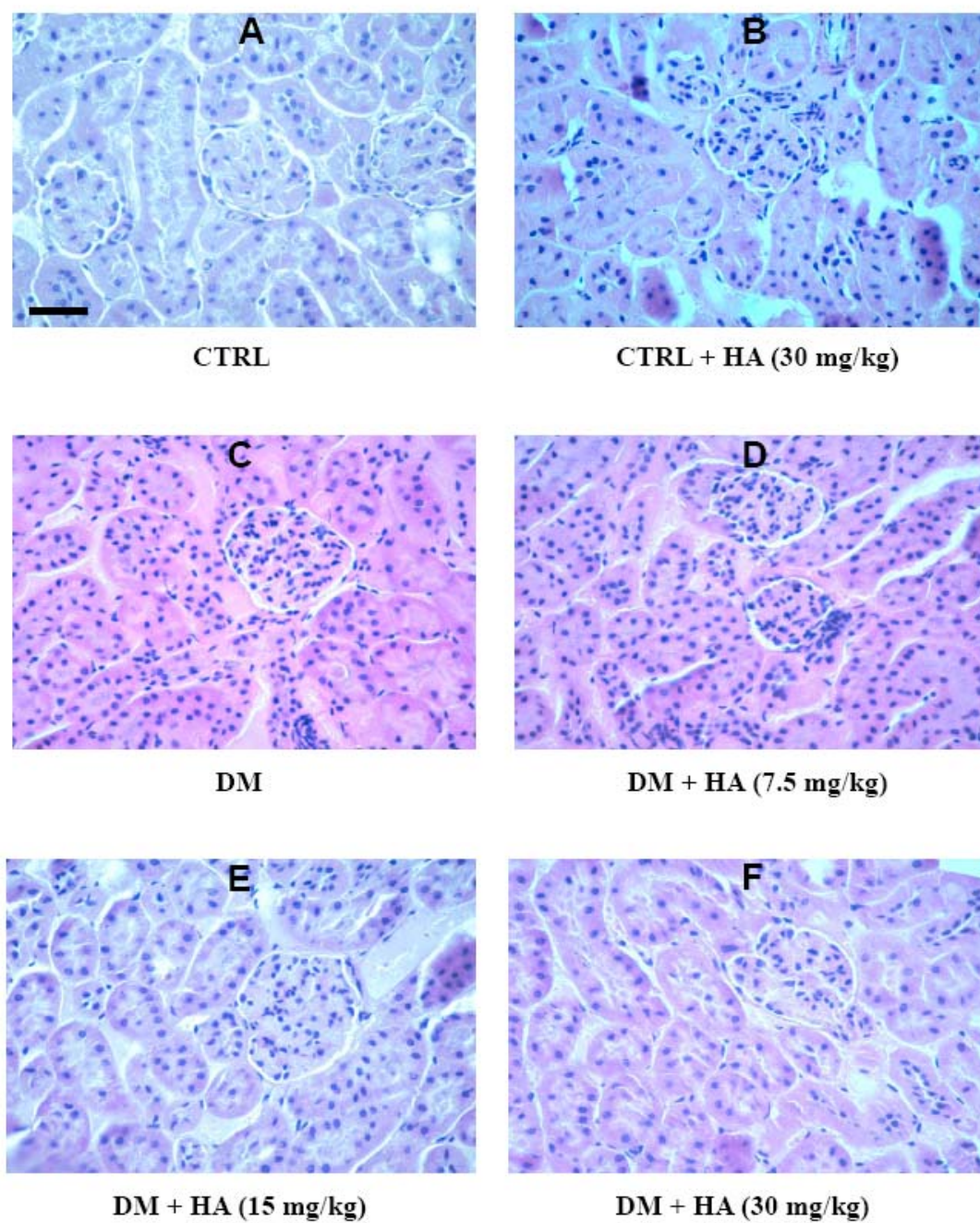


Fig. 11