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ORIGINAL ARTICLE

Efficacy of Hyaluronic Acid in the Treatment of MIA-induced Ankle Osteoarthritis in Rats and its Effect on Antioxidant Response Element

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Abstract
Ankle osteoarthritis (OA) is a long-standing inflammatory degeneration disease; until now, its pathogenesis remains ambiguous. There is no complete remedy from OA and the present pharmacological therapy choices are restrained and combined with undesirable side effects. Clinically, Hyaluronic acid (HA) is widely consumed to cure OA. The present experiment aimed to assess the role of HA in the remedy of experimentally Monosodium iodoacetate (MIA) -induced ankle OA in the rat model. Thirty male Wistar rats were divided into 3 groups (each of 10 rats). Rats of group I were injected with 1 mg MIA in the right ankle joint for two successive days, while those of group II were treated with saline instead of MIA; and group III(osteoarthritic + HA) rats were injected with HA in the ankle joint at 2nd, 3rd, and 4th weeks following injection of MIA. Bodyweight, ankle measurement, total leukocytes count (TLC), antioxidant response element (ARE) level, and joint Magnetic Resonance Imaging (MRI) were investigated. HA reduced expressions of joint ARE level. HA also markedly reduced the TLC. The administration of HA decreases ankle measurement in MIA-induced OA rats. MRI of HA showed a gradual reduction in joint damage. These results suggest that HA has improvement effects on OA rats which are assessed through anti-inflammatory and antioxidant effects.

Keywords Ankle osteoarthritis, Antioxidant response element, Hyaluronic acid

1. Introduction
Osteoarthritis (OA), is a disorder of the whole joint which is distinguished by its chronicity, deterioration of cartilage, and bone remolding, influences most of elder animal around the world **(Chen et al. 2017).** There are sequences of pathological alterations in OA, for instance, the synovial hyperplasia, sclerosis of the subchondral bone, and formation of osteophyte **(Kim et al. 2014).** OA is distinguished by the ongoing loss of cartilage conveyed by its clinical symptoms such as articular pain, deformity of joints, and activities of the normal joint become restricted. Therefore, OA will extremely lessen the quality of diseased animals and even results in incapacity. OA treatment, thus, is crucial and must undergo intense examination **(Liu et al. 2020).** Articular cartilage has featured composition and characteristic structure. It does not contain enough blood vessels, lymphoid tissues, or nerves to provide nutrition, and its nutrition supply depends chiefly on synovial fluid; too, chondrocytes of joints which are primarily hyaline chondrocytes, has a restricted capacity of proliferation **(Gomoll and**

Minas 2014). Hence, articular cartilage regeneration after injury or destruction is challenging. Even though usual treatments, such as surgical interventions, NSAIDs, intraarticular corticosteroid and cartilage tissue grafting, alleviate the signs of the disease to a certain degree, their remedy efficacy is not adequate **(Bruyère et al. 2014).** These routine therapies usually lessen pain, improve joint function, and diminish incapacity but do not make cartilage regeneration. Additionally, these treatments have side effects due to their chronic use ARE, replies basically to oxidative stress inducers. At oxidative stress status, transcription factor Nrf2 (NF-E2-related factor2) attaches to the ARE to enhance antioxidant genes activation. At normal condition and in the lack of main cellular stresses, NRF2 is connected to Keap1 in the cytoplasm. Once ROS overwhelms the capacity of the endogenous antioxidant, Keap 1 liberates NRF2 which then move to the nucleus where it unites ARE **(Bhakkiyalakshmi et al. 2018).** The signaling pathway of Nrf2/ARE is predominantly responsible for cellular defenses versus oxidative stress under the pathological states of OA. Expression of ARE is evident in the arthritic joints of mice but not clear and active in healthy joints **(Khan et al. 2018).** Route of drug administration in treatment of OA is crucial, Intra-articular administration reaches the cartilage and the synovial membrane, creating an alteration in the articular environment that sluggish the development of articular pain and arthritis and adjusts the clinical aspects **(Sánchez et al. 2016).** HA is an essential component of the function of the normal joint. It is an important particle in both the articular cartilage and the synovial fluid and where it supplies the support of the accumulating proteoglycan aggrecan **(Lalevée et al. 2016).** As a main constituent of the extracellular matrix, HA contributes in various biological activities such as proliferation of the cell, differentiation, and migration. Additionally, HA has been demonstrated to be essential in the cartilage tissue composition and synovial fluid **(Liu et al. 2020).** HA has been extensively consumed for stem differentiation into chondrocytes and damaged articular cartilage repair **(Toh et al. 2010).** Considering these factors, the purpose of the present investigation was to evaluate the efficacy of intraarticular administration of HA in improving inflammation and joint destruction caused by MIA in a rat model in order to find a better treatment for OA. Also in our study, we investigate the effect of HA on ARE.

2. Material and Methods

2.1. Animals

Thirty adult male Wistar rats, its weights ranged from 100 to 150 g, representing 8-10 weeks of age; rats were obtained from the laboratory animal unit of Helwan Farm-VACSERA, Egypt. Ten days prior the onset of the experiment, animals were kept under monitoring to preclude any inter current diseases. The animals dwelled in cages which made of polypropylene with covers of aerated stainless steel at (10-12h/day) natural daily lighting cycle and adjusted temperature (20-25°C), and were given a stabilized diet and water ad libitum.

2.2. Induction of osteoarthritis

Rats undergo anesthesia through the administration of 70 mg/kg ketamine, and7 mg/kg xylazine, OA was induced by intraarticular infiltration of 50 µL normal saline containing 2 mg Monosodium iodoacetate (2 mg/50µL) (Sigma-Aldrich, St. Louis, MO) with a 21 -gauge needle into the ankle joint of the right hind limb on 2 consecutive days, as previously illustrated by **Möller et al. (2019).**

2.3. Animal grouping

The prepared animals were divided into 3 groups randomly, each has 10 rats.

2.3.1. Normal group

Rats in the normal group were injected with 50µL saline in the right ankle joints.

2.3.2. Osteoarthritic group

Rats in the osteoarthritic group were injected with MIA in the right ankle joints for two successive days. Both of osteoarthritic and normal control rats were treated with 50µL saline in the right hind ankle joint at 2nd, 3rd, and 4th weeks after saline/MIA infiltration.

2.3.3. Osteoarthritic-HA group

Rats in the MIA-HA group were treated with 50 µL of 15mg/ml HA (orthovisc® (15mg/ml), (high molecular weight, molecular weight range 1000–2900 KDa; Anika Therapeutics, Inc. Bedford, USA) in the right ankle joints at 2nd, 3rd, and 4th weeks after injection of MIA. The body weight was measured at the beginning and end of the study to evaluate weight gain. At the end of experimental periods, Right ankle joint from each animal was extirpated after dissection. Ankles of each group were preserved at -20°C until consumed in the molecular test.

2.4. Ankle measurement

We assessed the changes in the transverse and anteroposterior diameters of the normal, diseased, and treated ankles. Ankle diameters were determined by micrometer **(Jimbo et al. 2017).** The measurements were taken every week (on the zero days till THE end of experiment) after THE injection of MIA.

2.5. Count of total leukocytes detection

Leukocytes count was assessed by using Turk's fluid Turk's solution that is composed of a stain (gentian violet and 1% acetic acid) **(Miale 1972).**

2.6. Magnetic resonance imaging

Two rats from the normal, MIA, and MIA-HA groups were at random selected for MRI imaging; before and after treatment, scanned by MRI after anesthetized the rats using an intraperitoneal injection of ketamine and xylazine (70 mg/kg ketamine, 7 mg/kg xylazine). The rats were scanned on a 1.5 Tesla whole-body MR scanner (Philip Medical System Intera) utilizing an extremity coil. The rats were positioned in the prone position with the hind legs extended caudolaterally by using tape to fix the rat so that the right ankle joint was placed in the middle of the scanning coil. MR images were acquired with a T1-weighted sequence in coronal slice orientation using the following sequence parameters: (TR=3000ms, TE=15ms, slice thickness= 2 mm).

2.7. Real-time PCR assay for antioxidant response element (ARE) mRNA of ankle joint

All the molecular analyses have been carried out at Laboratory of biochemistry department, Kasr Al Ainy Faculty of Medicine, Cairo University, Egypt Total RNA was isolated using Qiagen tissue extraction kit (Qiagen, USA) according to directives of manufacture. The total RNA (0.5–2μg) was used for cDNA conversion through high capacity cDNA reverse transcription kit Fermentas, USA).Real-time qPCR amplification and analysis were completed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets was adjusted at the annealing temperature. The primer sequence was exhibited in **Table (1).**

Table 1: The primer sequence of the examined gene

Target Gene	Primer sequence
ARE	Forward primer: 5'-TTGTAGATGACCATGAGTCGC-3' Reverse primer: 5'-TGTCCTGCTGTATGCTGCTT-3'

2.8. Statistical analysis

Statistical analysis was achieved by using SPSS v.25. Results were expressed as mean ± standard error (SE), all statistical comparisons were performed by analysis of Duncan's test post hoc. Values of $p< 0.05$ were deemed significant however those of $p> 0.05$ were deem non-significant.

3. Results

3.1. Effect of HA on the weight gain

The final body weight and body weight gain in normal control group, osteoarthritic group, and HA treated group are shown in **Table (2)** and **Fig. (1).** The gains in body weight were comparable between MIA- rats from normal, and HA treated group at zero-days and six weeks the osteoarthritic rats gained less weight than the normal rats. The osteoarthritic control rats exhibited an obvious decrease in the body weight in the 6th week when compared to the normal control group. HA treated rats gained more weight when compared with the osteoarthritic control group. The treatment of the osteoarthritic rats with HA produced a noticeable increase of the bodyweight at the end of the experiment when compared to the osteoarthritic control rats.

Table 2 Effect of HA treatment on body weight and weight gain in osteoarthritic rats

	Parameters		
	Initial body weight (g)	**Final body weight (g)**	**Weight gain (g)**
Normal group	119 ± 4[a]	201 ± 6[a]	82 ± 3[a]
Osteoarthritic control group	120 ± 4[a]	161 ± 2[c]	41 ± 2[c]
Osteoarthritic + HA group	121 ± 6[a]	183 ± 5[b]	62 ± 4[b]

Expressed data mean ± standard error. Number of noticed rats for each group is ten

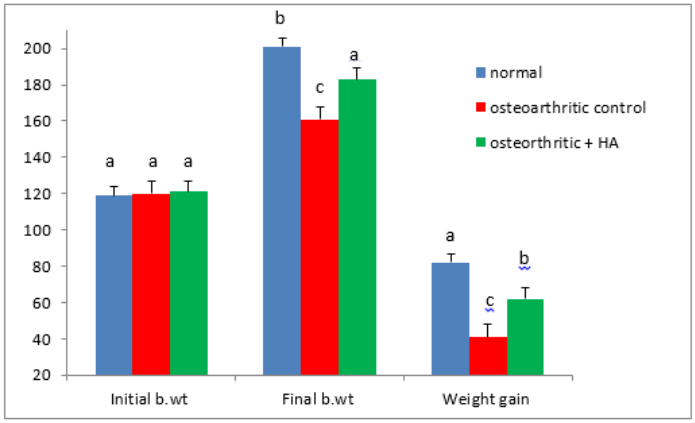

Fig. 1. HA treatment effect on body weight and weight gain in osteoarthritic rats.

3.2. Changes in ankle measurements

When compared with normal control rats, MIA rats had markedly swelling in the anteroposterior and transverse diameters of the right ankle at all post-injection time points **(Tables 3, 4).** While noticeable changes in ankle anteroposterior or transverse diameter were detected between MIA and MIA-HA rats at THE all-time points. Thus, HA injection yielded obvious effects on ankle appearance.

Table 3: Effect of HA treatment on ankle measurements (Anteroposterior) of osteoarthritic rats.

	Parameter (Anteroposterior) (mm)													
	Zero-day	**%**	**3rd day**	**%**	**2nd week**	**%**	**3rd week**	**%**	**4th week**	**%**	**5th week**	**%**	**6th week**	**%**
Normal group	**0.49± 0.01[a]**	**—**	**0.56± 0.01[b]**	**—**	**0.56± 0.01[b]**	**—**	**0.56± 0.01[c]**	**—**	**0.56± 0.01[b]**	**—**	**0.56± 0.01[d]**	**—**	**0.56± 0.01[d]**	**—**
Osteoarthritic control group	**0.50± 0.01[a]**	**2**	**0.75± 0.01[a]**	**34**	**0.67± 0.01[a]**	**21**	**0.66± 0.01[a]**	**17**	**0.67± 0.01[a]**	**20**	**0.65± 0.01[a]**	**17**	**0.66± 0.01[a]**	**18**
Osteoarthritic + HA group	**0.49± 0001[a]**	**-1**	**0.73± 0.02[a]**	**-2**	**0.68± 0.01[a]**	**2**	**0.64± 0.02[bc]**	**-2**	**0.63± 0.01[b]**	**-8**	**0.61± 0.01[c]**	**-9**	**0.59± 0.01[bc]**	**-7**

Expressed data mean ± standard error. Number of observed rats for each group is ten

3.3. Effect of HA on total leucocytic count

The total leukocytes count was extensively elevated in MIA-induced osteoarthritic group as compared with the normal one. The treatment of osteoarthritic rats with HA Produced a marked improvement in total leukocytes' count **(Table 5).**

3.4. MRI evaluation of OA

Magnetic resonance image of the normal ankle joint representing normal joint and foot anatomy **(Fig. 2A),** MR image of an osteoarthritic ankle joint after MIA injection revealed the augmented diameter of the joint and exhibited extensive soft tissue edema in acute osteoarthritic **(Fig. 2B)** and soft tissue edema reduced in chronic osteoarthritic **(Fig. 2C).** In contrast, HA treatment revealed mainly low signals and decreased diameter of the joint **(Fig. 2D),** demonstrating that HA is effective for suppression of inflammation and gives a good therapeutic outcome.

Table 4: Effect of HA treatment on ankle measurements (Transverse) in osteoarthritic rats.

	Parameter (Transverse) (mm)													
	Zero day	%	3rd day	%	2nd week	%	3rd week	%	4th week	%	5th week	%	6th week	%
Normal group	0.49± 0.01[a]	— -	0.56± 0.01[b]	— -	0.56± 0.01[b]	— -	0.56± 0.01[c]	— -	0.56± 0.01[b]	— -	0.56± 0.01[d]	— -	0.56± 0.01[d]	— -
Osteoarthritic control group	0.50± 0.01[a]	2	0.75± 0.01[a]	3 4	0.67± 0.01[a]	2 1	0.66± 0.01[a]	1 7	0.67± 0.01[a]	2 0	0.65± 0.01[a]	1 7	0.66± 0.01[a]	1 8
Osteoarthritic + HA group	0.49± 0001[a]	-1	0.73± 0.02[a]	-2	0.68± 0.01[a]	2	0.64± 0.02[bc]	-2	0.63± 0.01[b]	-8	0.61± 0.01[c]	-9	0.59± 0.01[bc]	-7

Table 5: Effect of HA treatment on TLC in osteoarthritic rats.

	Parameters	
	TLC (cell×103/mm3)	% change
Normal group	7.15 ± 0.4[c]	—
Osteoarthritic control group	16.5 ± 1.5[a]	131
Osteoarthritic + HA group	8.46 ± 0.9[b]	-48

Expressed data mean ± standard error. Number of assessed sample for each group is ten
Means, which take the similar superscript symbol (s), are not significantly altered. Percentage changes were estimated by comparing osteoarthritic control group with normal and osteoarthritic-treated groups with the osteoarthritic control one.

Table 6: Effect HA treatment on ARE mRNA expressions in joint tissue osteoarthritic rats.

	Parameters	
	ARE mRNA fold change (relative to control)	% change
Normal group	1.0 ± 0.1[c]	—
Osteoarthritic control group	5.5 ± 0.1[a]	448
Osteoarthritic + HA group	2.3 ± 0. 2[b]	-59

Expressed data mean ± standard error. Number of assessed joint for each group is three
Means, which take the similar superscript symbol (s), are not significantly altered. Percentage changes were estimated by comparing osteoarthritic control group with normal and osteoarthritic-treated groups with the osteoarthritic control one.

3.5. Effect on Joint ARE

Data illustrating the effect of HA on joint ARE mRNA expressions of osteoarthritic rats are shown in figures 3 and table 6. The administration of MIA to rats produced a significant ($P < 0.05$) increase in the expression level of ARE recording percentage of 448% in joint tissue as compared to the normal animals. The HA treated rats showed an obvious diminish in the elevated level of ARE expression as compared to the arthritic control group. Treatment of the osteoarthritic rats with HA noticeable reduction in the mRNA and protein level of ARE was still observed in HA group (change percentage -59%) comparing with that in MIA osteoarthritic rats.

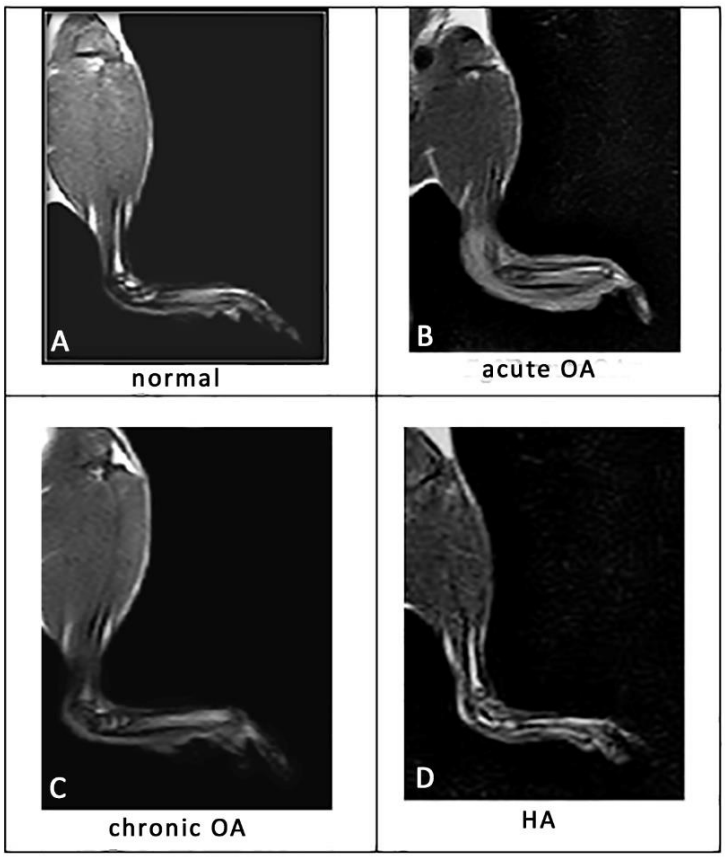

Fig. 2. T1-weighted MR images of the right ankle joints in normal, MIA and HA treated rats. Representing the normal joint and foot anatomy **(A)** MR image of an osteoarthritic ankle joint reveals the enlarged diameter of the joint and exhibits extensive soft tissue edema in acute osteoarthritic **(B)** and soft tissue edema reduced in chronic osteoarthritic **(C).** In contrast, HA treatment revealed diminished diameter of the joint **(D).**

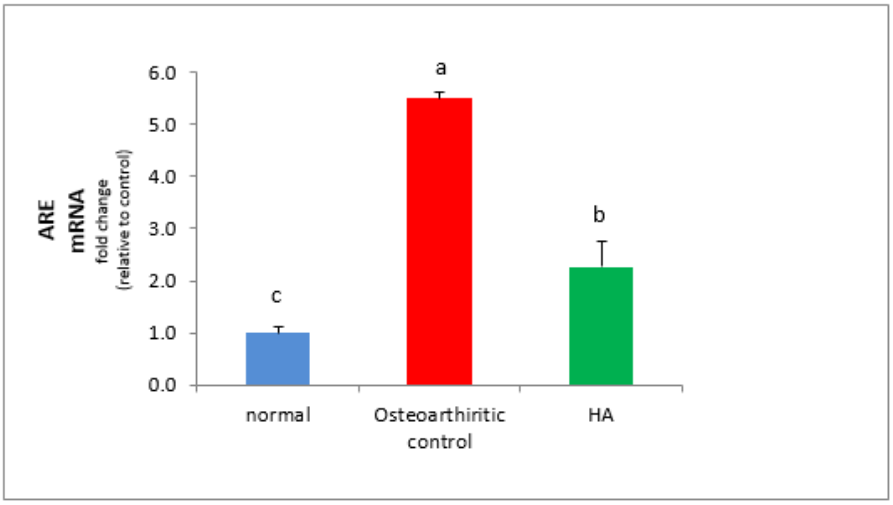

Fig. 3. Effect of HA treatment on ARE mRNA expression levels in joint tissue of osteoarthritic rat. The means, which have the same symbol (s), are not significantly different

4. Discussion

OA research has great attention in recent decades due to its high incidence, its clinical prognosis, and its effect on the health of the animal. Although OA prognosis is unsatisfactory now, and current treatments are not effective for the complete cure of OA. It is critical to determine an effective and simple therapy to treat OA in the clinic **(Liu et al. 2020).** In the rat OA model induced with MIA created in the

current experiment, we noticed extensive body weight loss. This result is in agreement with the previous study of **Jasemian et al. (2011)** who reported that Progressive lessening of bodyweight gain has been achieved between arthritic animals during arthritis development. Also, **Lee et al. (2014**) reported that The MIA injection resulted in a markedly reduced in weight gain compared with normal rats. The curative effect in the HA treated group exhibit higher weight gain than the MIA group in the 5th and 6th weeks. The rate of weight gain increased during this experimental period, suggesting that the animals were underneath low stress and/or in low pain. In the present study, noticeable increases in both the right ankle anteroposterior and transverse diameters in all of osteoarthritic rats relative to the normal control group were observed at all points of observation. These findings are in a line with those of **Jimbo et al. 2017** who recorded that MIA elevates the ankle anteroposterior and transverse diameters. The treatment of the osteoarthritic rats with HA produced deteriorate of the elevated level of the right ankle anteroposterior and transverse diameters at the end of the experiment relative to the osteoarthritic rats. This is like the previous results reported that intra-articular HA significantly diminished OA severity and mechanical hyperalgesia of the paw **Ikeuchi et al. (2015**). Also, **Altman et al. (2019)** recorded that intra-articular of HA injection is frequently used to reduce inflammation and stimulate regeneration. On the other hand, **Jimbo et al. (2017**) reported that there is no significant alterations in ankle anteroposterior or transverse diameter were noticed among MIA and MIA-HA rats at any time point. Over the previous years, the diagnostic utility of MRI in arthritis investigation has advanced from a technique with the potential to one of application for the noninvasive picturing of soft tissue and bony changes in arthritic joints (**Jacobson et al. 1999**). In our study, magnets with low field strength 1.5 Tesla, resulting in poor anatomic resolution. Added shortage involved an inability to illustrate the underlying pathology contributing to changes in hydrogen content in arthritic joints. The final aim of this study was to evaluate the MRI data in the circumstance of a variety of physiologic (bodyweight, ankle measurement), biochemical (ARE level), and cellular (leukocyte counts) parameters. This study was not aimed at determining if MRI could substitute for anyone index of illness progress, but to determine if alterations in MRI images could be linked with any other systemic signs. In our study, rises in signal intensity of MRI in the right hind paw closely reflected increases in ankle measurement, and leucocytic counts. These former inflammatory responses peaked between days 3 and 14 after the administration of MIA. After the treatment of osteoarthritic rats with HA, the intensity of the signals of MRI diminish from 4th week onward, parallel to the observed decrease in ankle measurement, indicating that the inflammatory response was in a decrease in HA treated group. The TLC data exhibited an extreme leukocytosis in the osteoarthritic rats. This leukocytosis is referred to inflammation induced by monosodium iodoacetate **(Bahtiar et al. 2017).** In the current study, it was found that the HA declined markedly the elevated TLC near to their normal levels, indicating the anti-inflammatory effect of HA therapy on the MIA course. In the same way **Aniss et al. (2020)** revealed that HA has an anti-inflammatory effect which is mainly related to reduce leukocyte count. Recent studies have proposed an oxidative stress role in the pathogenesis of OA. Oxidative stress is produced constantly, affecting cells and the extracellular matrix. High levels of ROS, in combination with the antioxidant reduction, indicate disease progress (**Yamada et al. 2019**). It responds mostly to oxidative stress inducers. Under conditions of oxidative stress, Nrf2 binds to the ARE to activate antioxidant genes (**Bhakkiyalakshmi et al. 2018).** In MIA osteoarthritic control rats, joint ARE mRNA expression was markedly expressed when compared to the normal rats. This observation is in line with the study of **Cai et al. (2015)** who found that ARE is elevated in the joints of mice with MIA-OA. The increase in ARE, which happens in mRNA expression was expressed as compared to the normal rats. As unstressed cells have a very low level of Nrf2 protein, these results suggest that an oxidative stress-induced Nrf2 activation occurs during OA. In the present work, we show that treatment of the osteoarthritic rats with HA a significant reduction in the mRNA and protein level of ARE was observed in HA group, compared with that in the osteoarthritic rats. These results prove that these treatments have shown to play a fundamental role in defense against oxidative injury through the motivation of the Nrf2/ ARE signaling pathway in cartilage.

5. Conclusion

In conclusion, we demonstrate, that intra-articular injection of (50μL) of HA can improve osteoarthritis in a MIA induced OA in the rat model. The curative effect can be attributed to the subsequent reasons: anti-inflammatory effect (diminish Leucocytic count) and antioxidant effect (lessen the mRNA and protein

level of ARE). We consequently believe that HA is a hopeful solution to the degeneration of articular tissues and achievement clinical treatment is potential.

6. Conflict of interest

There is no conflict of interest to declare

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