

THE EFFECT OF INTRA-ARTICULAR INJECTIONS OF HIGH MOLECULAR WEIGHT HYALURONIC ACID, BOTOX, AND THEIR COMBINATION ON COMPLETE FREUND'S ADJUVANT-INDUCED TEMPOROMANDIBULAR JOINT OSTEOARTHRITIS IN RATS. (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Introduction: Hyaluronic acid (HA) is the major hydrodynamic component of synovial fluid. Thus, intra-articular injection of HA (IA-HA) has become the most widely accepted therapy for osteoarthritis. Growing interest has been seen recently in new uses for Botox in medicine, such as the treatment of osteoarthritis.

Aim of the work: This study was carried to investigate the effect of intra-articular injection of Botox, Hyaluronic acid and their combination on complete Freund's adjuvant (CFA) induced temporomandibular joint osteoarthritis (TMJ-OA) in rats.

Materials and Methods: 30 young adult male Albino rats weighing 250–300g were used in this study. Rats were divided randomly into 3 groups. Group I (control) in which animals received 3 doses of saline on days 7, 14 and 28. Group II (osteoarthritic) in which animals received single dose of CFA and left without treatment. Group III (treatment) was subdivided into 3 subgroups. In each subgroup, animals received 3 doses of high molecular weight hyaluronic acid in subgroup A, Botox in subgroup B, and combination of both in subgroup C on days 7, 14, and 28. After 35 days, the animals were sacrificed and the TMJs were dissected and examined histologically and immunohistochemically for tumor necrosis factor alpha.

Results: TMJ retained its normal histological structure after IA-HA. There was no improvement in osteoarthritic features by intra-articular injection of Botox. The combination improved osteoarthritic features more than Botox but did not reach the level of HA.

Conclusion: TMJ-OA could be treated with IA-HA, whereas Botox and their combination were ineffective.

KEYWORDS: TMJ-osteoarthritis, Hyaluronic acid, Botox

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INTRODUCTION

Temporomandibular joint osteoarthritis (TMJ-OA) is often a slowly progressing, asymmetric disease that destroys the articular tissues. It manifests as functional pain, crepitus, or other joint noises. Progressive cartilage breakdown, chondrocyte death, and subchondral bone remodeling are the main pathological changes associated with TMJ-OA. The causes of TMJ-OA are complex, multifactorial, or unknown^[1].

TMJ-OA is treated using non-invasive techniques which are physical therapy, occlusal splints, and non-steroidal anti-inflammatory medications. Arthrocentesis and/or intra-articular injection are examples of minimally invasive procedures. Surgical techniques include arthroplasty and condylectomy^[2].

Hyaluronic acid (HA) is a polysaccharide of the glycosaminoglycan family that can be found in many extracellular tissues, including synovial fluid and cartilage^[3]. In the joints, synoviocytes and chondrocytes produce HA. HA acts as a lubricant, absorbs shock, and distributes mechanical forces impacting the joint. Depolymerization of HA in osteoarthritis patients lowers its molecular weight and viscoelasticity. Due to these changes, cartilage damage is more likely to occur^[4].

Rydell and Balazs described the first medical application of intra-articular injections of sodium hyaluronate for the treatment of knee osteoarthritis in 1971^[5]. It has been suggested as a treatment for TMJ-OA^[6,7]. It was shown that intra-articular injections of HA minimize friction and stress on the joint cartilage by preserving TMJ homeostasis and contributing to lubrication, chondroprotection, and proteoglycan production. Additionally, it has a significant impact on relieving TMJ-OA symptoms through anti-inflammatory activities by lowering pro-inflammatory mediator generation and activity^[8-10].

Botox (BTX), a minimally invasive technique, has recently attracted a lot of interest in the dental field due to its advantages in treating a variety

of oral diseases such as temporomandibular disorders (TMDs), bruxism, mandibular spasm, and masseteric hypertrophy^[11]. The neurotoxin BTX, which is produced by the bacterium *Clostridium botulinum*, blocks the neuromuscular junction and prevents acetylcholine from being released into the synaptic cleft. This results in chemical denervation, which finally leads to flaccid paralysis^[12].

In this study, albino rats with induced TMJ-OA were treated using two non-invasive regimes of treatments, HA and Botox, as well as a combination of both. The goal was to determine the anti-inflammatory effect and regenerative potential of these regimens.

MATERIALS AND METHODS

30 young adult male Albino rats weighing 250–300g were used in this study. The animals were housed in clean metal cages at the faculty of medicine at Minya University under optimal conditions. They had free access to food and ad libitum water for the whole experimental period.

All experiments were carried out in accordance with research protocols established by the animal care committee of the National Research Center, Egypt, which follows the recommendation of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Technique for intra-articular injection of TMJ

The TMJ was felt 5–10 mm posterior to the lateral canthus of the eye, and the mandible and the zygomatic arch of the temporal bone in front of the ear were also felt. The superior joint cavity was reached by placing the sterile 30-gauge needle attached to a 1 ml plastic insulin syringe on the posteroinferior border of the zygomatic arch (the roof of the joint fossa) and passing it through the posterolateral edge of the mandibular condyle in an antero-medial orientation^[13].

Prior to injection, each animal was given a diethyl ether anesthetic. The animals were divided randomly into three groups:

Experimental design:

Group I (control group): consisted of 6 rats which received 3 intra-articular injections of 50 μ l of saline into upper compartment of the left TMJs on days 7, 14, and 28.

Induction of TMJ-OA

Induction of OA at day 0 in group II and group III by single intra-articular injection of (50 μ l of complete Freund's adjuvant (CFA) (5881; Sigma-Aldrich, USA), diluted 1:1 (oil: saline) ^[14] into the upper compartment of left TMJ. After OA induction, the animals received 300 mg/kg of acetaminophen (Paracetamol) added to drinking water to alleviate pain ^[15]. Each study animal's contralateral right TMJ was left unaltered in order to preserve animal feeding ^[16].

By monitoring an increase in temperature in the region around the left TMJ of the CFA-injected rats the day after the injection, inflammation was confirmed. The temperature was contrasted with that of the TMJ on the opposite side ^[14].

- Group II (osteoarthritic group): consisted of 6 rats where rats were left without treatment.
- Group III (treatment group): consisted of 18 rats. These rats were divided into 3 subgroups, and then rats in each subgroup received the treatment.

Subgroup A: Hyaluronic acid group (HA): consisted of 6 rats. Each rat received 3 intra-articular injections of 50 μ l high molecular weight hyaluronic acid (HMWHA) (2.3–2.5 million Daltons) [16] (Optivisc, UK) into the upper compartment of the left TMJs on days 7, 14 and 28 after induction of osteoarthritis.

Subgroup B: Botox group (BTX): consisted of 6 rats. Each rat received 3 intra-articular injections (of 5 units/kg of Botox diluted into 50 μ l of 0.9 NaCl solution) ^[17] into the upper compartment of

left TMJs on days 7, 14 and 28 after induction of osteoarthritis.

Subgroup C: combination group: consisted of 6 rats. Each rat received 3 intra-articular injections of a combination of (HMWHA and Botox) into the upper compartment of left TMJs on days 7, 14, and 28 after induction of osteoarthritis.

At 35 days, all animals were sacrificed by diethyl ether overdose, and their TMJs were dissected for histological and immunohistochemical study.

Histological preparation

The left half of the skull was immersed in 10% neutral buffered formalin. Then the samples were decalcified in a microwave oven at pH 7.8 with a 10% ethylene diaminetetracetic acid (EDTA) solution. Serial TMJ sagittal sections (5 μ m) were then achieved and stained with Hematoxylin and eosin ^[18].

Immunohistochemistry:

For immunohistochemical staining with TNF- α , steps were followed according to Chu et al., 1991 ^[19].

Image analysis

1. Condylar cartilage thickness measurement:

The thickness of condylar cartilage was measured at the middle portion, which included all zones of the condylar cartilage (fibrous, proliferative, fibrocartilage, and calcified).

Image J 22 software was used to analyze images for condylar cartilage thickness. Standard measuring frames per 6 photomicrographs for each group were transferred to the monitored screen using a magnification of x4.

Measurement procedures for condylar cartilage thickness (fig. 1):

1. A straight line tool was chosen to draw a line corresponding to the thickness of the condylar cartilage

2. The unit of measurement for length was the centimeter.
3. The distance in pixels field was automatically filled in based on the length of the line selection.

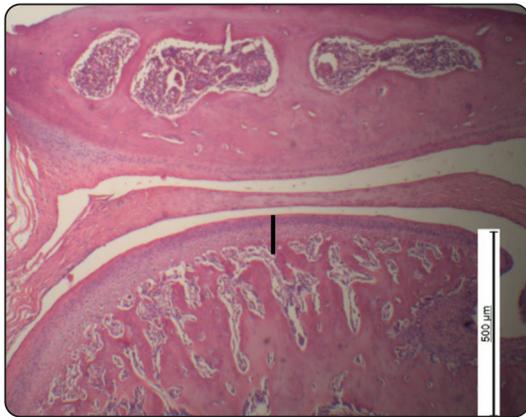


Fig. (1) Photomicrograph of the TMJ showing the locations where thickness was measured in the articular cartilage Mag. X4.

2. The number of TNF- α immuno-positive chondrocytes:

TNF- α immuno-positive chondrocytes in articular cartilage were counted using the software on 6 photomicrographs for each group at a magnification of x40.

Statistical Analysis

Data were verified, coded by the researcher, and analyzed using IBM-SPSS 24.0 (IBM-SPSS Inc., Chicago, IL, USA)*. Descriptive statistics: Means, standard deviations, median, inter-quartile range (IQR) were calculated. Shapiro-Wilk test would be used to test for data normality. Test of significances: For continuous variables with more than two categories; one-way ANOVA test was calculated to test the mean differences of the data that follow normal distribution, post-hoc test was calculated using Bonferroni corrections for pairwise comparisons between the study groups. A significant p-value was considered when it is less than 0.05.

RESULTS

1. The histological results using Hematoxylin and eosin

1. The control group: (Group I)

TMJ in the control group revealed the normal structural components of rat TMJ, including the condyle, articular disc, and temporal bone. Condyle had normal anatomy and contours. A fibrous layer, a proliferative layer, a fibrocartilaginous zone, and a calcified cartilage zone covered its articular surface. The subchondral bone beneath the cartilage displayed typical, interconnected bone trabeculae and little bone marrow cavities. (Fig.2 A and B)

2. Osteoarthritic group: (group II)

In the OA group, the thickness of articular cartilage was significantly reduced compared with group I ($p < 0.05$) (Table 1). Chondrocytes exhibited decreases in number, clustering, apoptosis and hypertrophic. Resorption in the Subchondral bone and temporal bone was associated with expanded bone marrow cavities infiltrated with inflammatory cells and accumulations of fat cells (Fig.2 C and D)

3. Treatment group: (group III)

Subgroup A: Hyaluronic acid group

In the HA group, TMJ retained its normal histological structure. Articular cartilage thickness was significantly increased compared with group II, subgroup B, and subgroup C ($p < 0.05$) (Table 1). Chondrocytes appeared with normal distribution, density, and morphology. Inflammatory cells and fat cells were reduced compared with group II, subgroup B, and subgroup C (Fig.3 A and B)

Subgroup B: Botox group

In the Botox group, the thickness of the articular cartilage was significantly reduced compared with the group I, subgroup A and subgroup C ($p < 0.05$), and insignificantly compared with the group II ($p > 0.05$) (Table 1). Chondrocytes exhibited clustering and

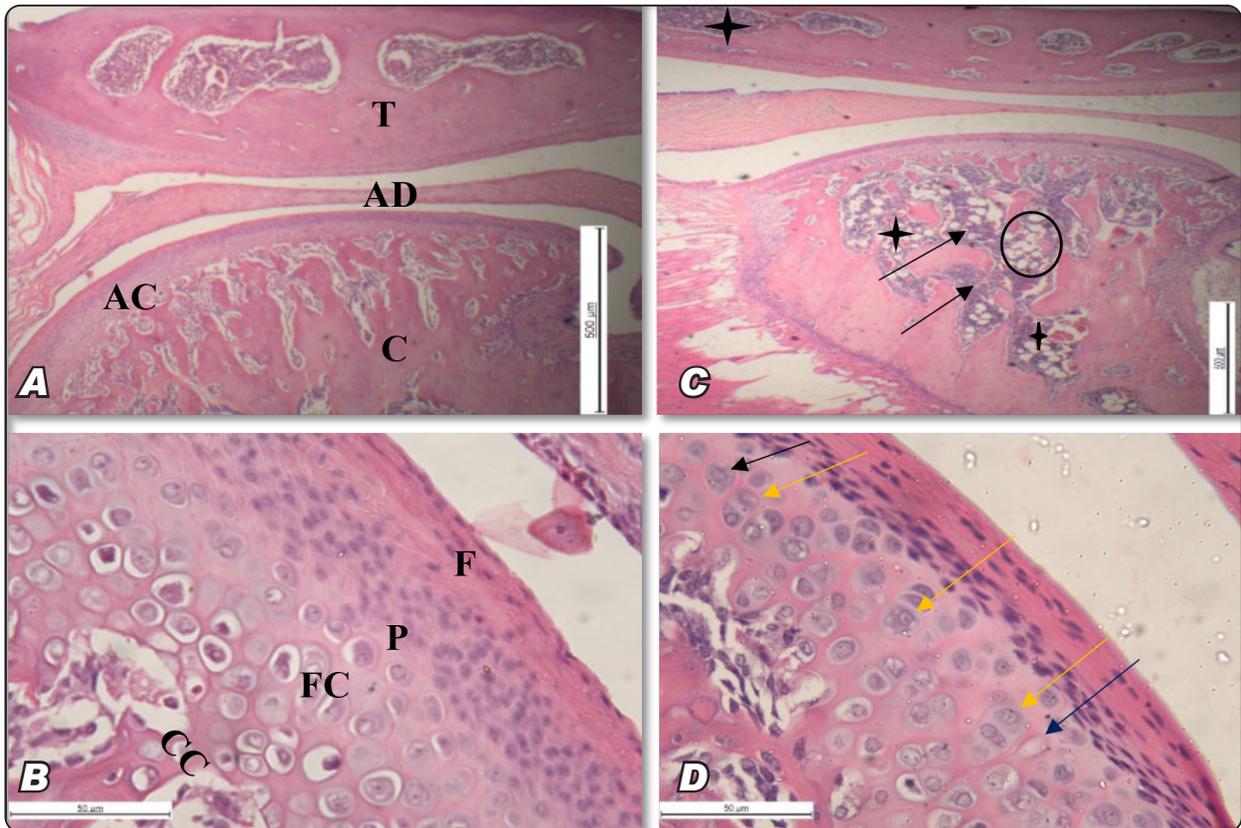


Fig. (2) Photomicrographs of sagittal section in TMJ from control group figs. (A, B), OA Group figs. (C, D) H&E staining: (A) TMJ revealed: normal TMJ configuration with normal temporal bone (T), intra-articular disc (AD), articular cartilage (AC) and condyle (C) (Mag.x4, scale bar 500 μ m). (B) Condyle with its fibrous covering (F), proliferative layer (P), fibrocartilaginous zone (FC), calcified cartilage (CC) (Mag.x40, scale bar 50 μ m). (C) TMJ revealed: expanded eroded bone marrow cavities filled with inflammatory cells in both temporal and subchondral bones (stars), accumulation of fat cells (circle) (Mag.x4, scale bar 500 μ m). (D) condyle showed: clustering chondrocytes (yellow arrows), hypertrophic chondrocytes (black arrow), empty lacuna (blue arrow) (Mag.x40, scale bar 50 μ m)

apoptosis. Bony trabeculae became sparser, thinner, and more discontinuous with osteoclastic activity and reversal lines. There was a greater infiltration of inflammatory cells and accumulation of fat cells in the bone marrow cavities of subchondral and temporal bones than in the other groups (Fig.3 C and D).

Subgroup C: Combination group

In the combination group, the thickness of the articular cartilage increased when compared to the group II, which was statistically insignificant ($p>0.05$), and the Botox group, which was statistically significant ($p<0.05$) (Table 1). Clustering, hypertrophy, and an empty lacuna can all be seen in chondrocytes. There was improvement

in temporal and subchondral bones and decreased infiltration of inflammatory cells and fat cells in bone marrow cavities compared with the Botox group (Fig.3 E and F).

Immunohistochemical results:

Immunohistochemical sections of the TMJ condyle from the control group and HA group revealed mild expression of the TNF- α marker in chondrocytes and the extracellular matrix (ECM). In the OA and Botox groups, the TNF- α marker was highly expressed in chondrocytes and moderately expressed in the ECM. In the combination group, the TNF- α marker was highly expressed in chondrocytes and ECM (Fig.3 & 4).

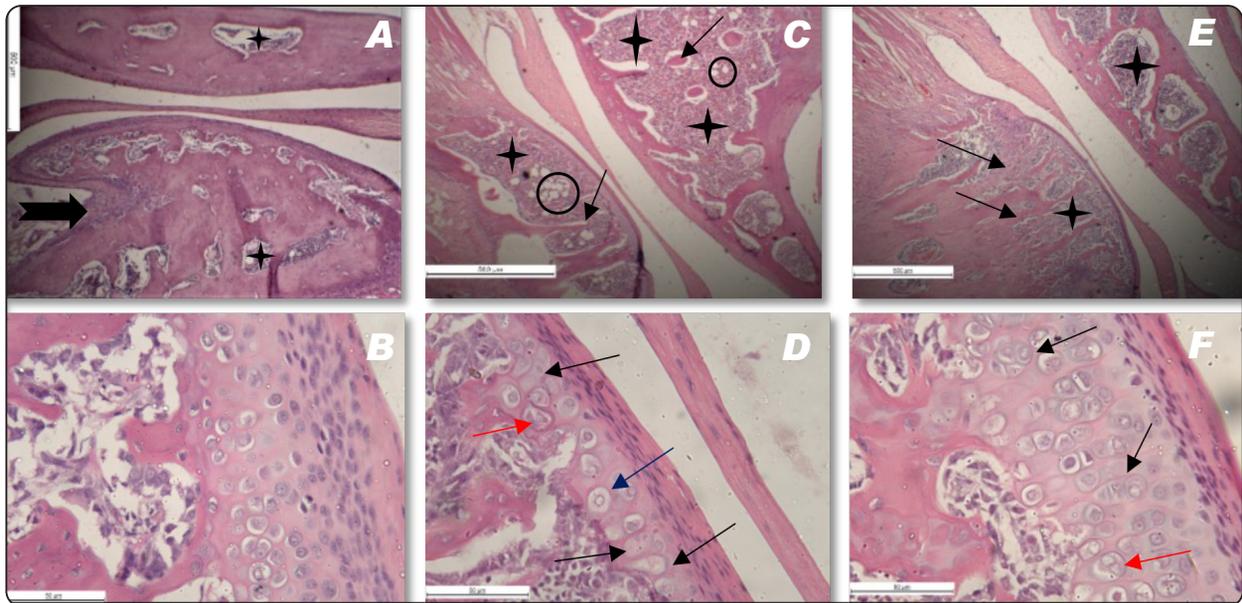


Fig. (3) Photomicrographs of sagittal section in TMJ from HA group figs. (A, B), Botox group figs. (C, D) & combination group figs. (E, F) H&E staining: (A) TMJ revealed: few bone marrow cavities filled with inflammatory cells in subchondral and temporal bones (stars), lateral fibrillation (notched arrow) (Mag.x4, scale bar 500 μ m). (B) condyle showed: chondrocytes with normal morphology and distribution, bone trabeculae enclosing marrow cavities (Mag.x40, scale bar 50 μ m). (C) TMJ showed: excessive expansion of bone marrow cavities filled with inflammatory cells in temporal bone & subchondral bones (stars), accumulation of fat cells (circles), thinner & sparse bony trabeculae (arrows) (Mag.x4, scale bar 500 μ m). (D) condyle showed: clustering of chondrocytes (red arrow), empty lacunae (black arrows), apoptosis of chondrocytes (blue arrow) (Mag.x40, scale bar 50 μ m). (E) TMJ revealed: numerous bone marrow cavities infiltrated with inflammatory cells (stars), regeneration of subchondral bone (arrows) (Mag.x4, scale bar 500 μ m). (F) condyle showed: clustering of chondrocytes (black arrows), apoptosis of chondrocytes (red arrow) (Mag.x40, scale bar 50 μ m).

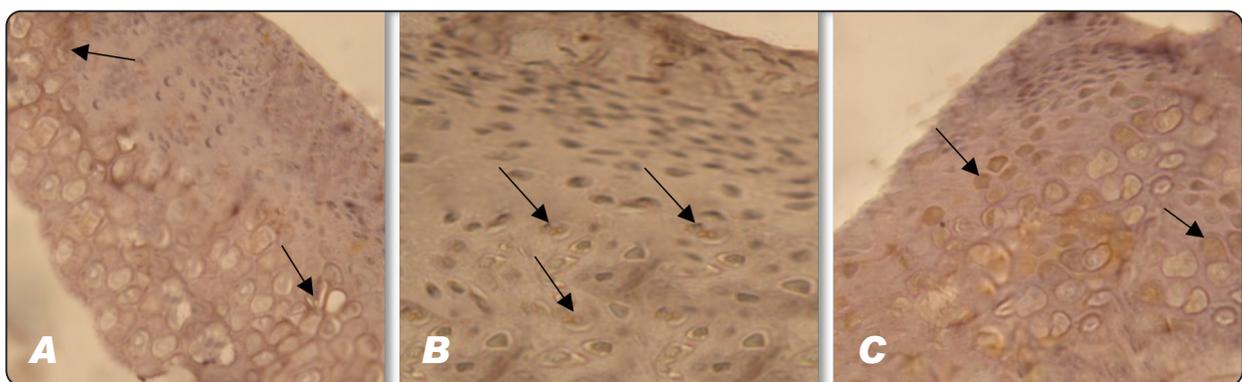


Fig. (4) Photomicrographs of immunohistochemical of TNF- α marker in articular cartilage from control group (A), OA group (B) & HA group (C). (A) showed: mild expression of TNF- α in chondrocytes and ECM. (B) showed: severe expression of TNF- α in chondrocytes and moderate in ECM. (C) showed: mild expression of TNF- α in chondrocytes and ECM, figs. 4 (A, B & C) immuno-positive chondrocytes (arrows) (Mag.x40).

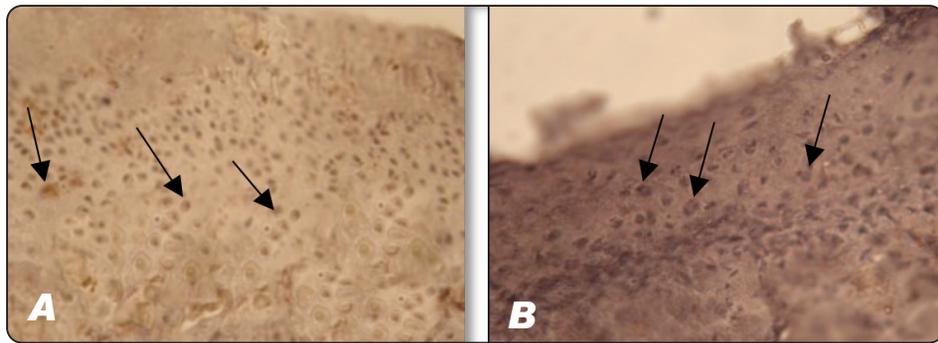


Fig. (5) Photomicrographs of immunohistochemical of TNF-α marker in articular cartilage from Botox group (A), combination group (B). (A) sever expression of TNF-α in chondrocytes and moderate in ECM. (B) sever expression of TNF-α in chondrocytes and ECM, figs. 5 (A & B) immuno-positive chondrocytes (arrows) (Mag.x40)

Statistical results:

The number of immuno-positive chondrocytes in articular cartilage:

The number of immuno-positive chondrocytes of articular cartilage in TMJ was significantly lower in group I (3.00 ± 0.8) compared with group II (54.50 ± 13.9), subgroup B (62.00 ± 15.1), and subgroup C (35.00 ± 18.1) (p <0.05), and insignificantly lower compared with subgroup A (5.25 ±

2.2) (p > 0.05). In group II (54.50 ± 13.9), it was significantly higher compared with subgroup A (5.25 ± 2.2) and subgroup C (35.00 ± 18.1) (p <0.05). In subgroup A (5.25 ± 2.2), it was significantly lower compared with subgroup B (62.00 ± 15.1) and subgroup C (p <0.05). In subgroup C (35.00 ± 18.1), it was significantly lower compared with subgroup B (62.00 ± 15.1) (p < 0.05). In subgroup B (62.00 ± 15.1), was insignificantly higher compared with group II (54.50 ± 13.9) (p > 0.05). (**Table 2 & Box-whisker 2**)

Image analysis results:

TABLE (1) comparison between groups as regard means ± SD of the articular cartilage thickness in different groups (significant *)

	Thickness of Articular Cartilage					P-value*
	Control (I)	OA (II)	HA (subgroup A)	Botox (subgroup B)	Combination (subgroup C)	
Thickness of Articular Cartilage						
• Mean ± SD	3.48 ± 0.9	1.84 ± 0.5	3.39 ± 0.7	1.05 ± 0.2	2.19 ± 0.2	= 0.001
• Median (IQR)	3.5 (1.5)	1.9 (0.9)	3.5 (1.3)	1 (0.4)	2.1 (0.4)	
P-value**	I vs II = 0.001*	II vs subgroup A = 0.002*	Subgroup A vs subgroup B < 0.001*	I vs subgroup B < 0.001*	I vs subgroup C = 0.006*	
	I vs subgroup A = 0.826	II vs subgroup B = 0.071	Subgroup A vs subgroup C = 0.009*	Subgroup C vs subgroup B = 0.013*	II vs subgroup c = 0.399	

* Kruskal Wallis test was used to compare the median difference between groups

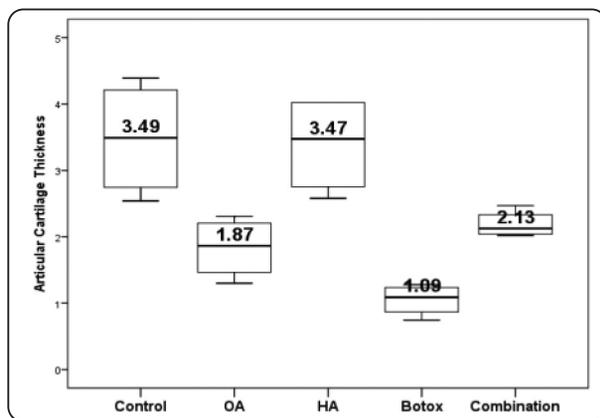
**Post-hoc test with Bonferroni Corrections was used to compare the mean difference between groups

TABLE (2) Comparison between groups as regard means \pm SD of the number of TNF- α positive chondrocytes in the articular cartilage in different groups (significant *).

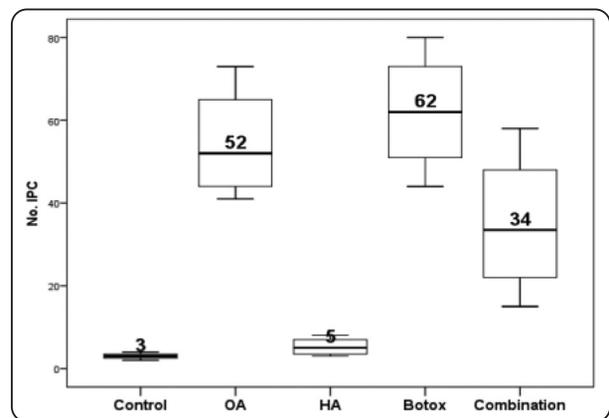
	No. Immuno- Positive Chondrocytes					P-value*
	Control (I)	OA (II)	HA (subgroup A)	Botox (subgroup B)	Combination (subgroup C)	
IPC No.						
• Mean \pm SD	3.00 \pm 0.8	54.50 \pm 13.9	5.25 \pm 2.2	62.00 \pm 15.1	35.00 \pm 18.1	
• Median (IQR)	3 (2)	52 (27)	5 (4)	62 (29)	33.5 (25)	
P-value**	I vs II < 0.001*	II vs subgroup A < 0.001*	Subgroup A vs subgroup B < 0.001*	I vs subgroup B < 0.001*	I vs subgroup C = 0.002*	< 0.001
	I vs subgroup A = 0.799	II vs subgroup B = 0.401	Subgroup A vs subgroup C = 0.004*	Subgroup C vs subgroup B = 0.007*	II vs subgroup C = 0.040*	

* *Kruskal Wallis test was used to compare the median difference between groups*

***Post-hoc test with Bonferroni Corrections was used to compare the mean difference between groups*



Box & Whisker (1) Differences in articular cartilage thickness of the TMJ between the studied groups.



Box & Whisker (2) Comparison between groups as regard means of the number of TNF- α positive chondrocytes in the articular cartilage in different groups

DISCUSSION

TMJ-OA is a progressive degenerative joint disease marked by inflammation, articular cartilage degradation, afferent nerve hypersensitivity, and subsequent chronic inflammatory pain [20].

In this study, TMJ-OA was induced by a single intra-articular injection of complete Freund’s adjuvant (CFA) 50 μ l. CFA is an inactivated dried mycobacteria that stimulates cell-mediated immunity, which leads to an increase in the

synthesis of certain immune-globulins [21, 22]. The current study’s time frame was chosen to be 35 days because induced TMJ arthritis utilizing CFA has been shown to persist for 6 weeks with higher inflammatory mediator concentrations [23].

Rats were chosen because of their easy handling during OA induction, quick skeletal maturity compared to larger animals, and similar condylar translatory movements to those of the human condyle [24].

In the OA group, the results revealed that the number of chondrocytes and the thickness of the condylar cartilage gradually decreased, while the bone marrow cavities were found to be expanded and the bone trabeculae were disorganized. The results agree with **Xu et al. 2016**, whose findings were attributed to increased Receptor activator of nuclear factor kappa-B ligand (RANKL) and decreased osteoprotegerin (OPG) expression in the chondrocytes. The findings resulted in cartilage and subchondral bone degeneration. Chondrocyte numbers were found to be decreased, in line with thinner cartilage and degraded proteoglycans^[25].

Regarding clusters of chondrocytes in the articular cartilage, **Soliman 2012**, investigated the effect of high- and low-molecular-weight hyaluronic acid on induced knee joint osteoarthritis. He found that cell clusters have been identified to express matrix-degrading enzymes and to be a significant source of pathogenic inflammatory mediators in OA. Chondrocyte cluster development is regarded as a distinctive feature of OA^[26].

In the present study, inflammatory cells were found to infiltrate expanded bone marrow cavities. This finding was consistent with those of **Chen et al. 2020**, who found inflammatory cell infiltration and an increase in inflammatory mediators using an ELISA assay. They also found bone marrow degeneration and articular cartilage degeneration^[27].

The result in the HA group revealed that the TMJ returned to its normal histological structure. This result agrees with **Tolba et al. 2019**, who found that three intra-articular injections of HMWHA into TMJ-OA in rats restored distinct structural components of the condyle and articular disc^[14].

The findings of this study came in line with **Duygu et al. 2011**, who found that in early stage TMJ-OA, HMWHA (Hylan G-F 20) reduced cartilage alterations, and chondrocyte clustering, demonstrating the chondroprotective benefits of Hylan G-F 20^[28].

Li et al. 2012, reported that the potential protective function of HMWHA may be explained by its capacity to regulate the synovial fluid's viscosity and lubricating characteristics, acting as a barrier between the synovial membrane and the articular surfaces^[29]. **Kim et al. 2001**, also reported that the cartilage layer had increased. The HA keeps things lubricated, which reduces wear and tear. Metabolically, HA contributes to the feeding of the disc's avascular regions and the condylar cartilage^[30].

Chakravarti et al. 2009, offered an explanation for these findings. They emphasized that HA treatment reduced the number of synovial RANKL positive cells because higher RANKL positive cells and lower OPG led to cartilage degeneration^[31]

Reduction of inflammatory cells infiltrate in the HA group compared with the OA group, agrees with **Lemos et al. 2015**^[32]. **Williams et al. 2007**, explained the anti-inflammatory action of HA when injected into osteoarthritic joints. HA of different MWs reduces interleukin-1, prostaglandin E2 (PGE2), and TNF α , scavenges free oxygen radicals, and attenuates matrix metalloproteinases-3 (MMP3), all of which found to reduce inflammation^[33].

In the Botox group, results revealed that the thickness of the articular cartilage and the number of chondrocytes were reduced. Apoptotic chondrocytes and empty lacunae were also detected in this study. These results agree with **Mohammed 2019**, who investigated the effects of a single injection of Botox into the masseter and temporalis muscles on the articular cartilage of the condyle in rabbits. She reported decreased articular cartilage thickness and condylar regeneration. In the hypertrophic zone, the majority of chondrocyte lacunae were empty. The usual architecture and morphology of chondrocytes in the proliferative and maturative zones has changed with the complete loss of their nuclei^[34].

The cartilage response in the Botox group of this study also emphasizes the findings of **Yaltrk et al. 2018** who investigated the effect of intra-articular

injection of Botulinum Toxin A into monosodium iodoacetate (MIA) induced TMJ-OA in rats and found cartilage lesions in the form of cell loss^[17].

On the other hand, **Namazi 2006**, who investigated the effect of intra-articular injection of botulinum toxin into the knee-OA joint in rabbits, found that botulinum toxin prevented osteoarthritis from progressing in its early stages, through its chondroprotective effects^[35].

Regarding bone response in the Botox group of the foregoing study, **Ail et al. (2018)** reported similar findings in the form of thin sparse discontinuous bone trabeculae with osteoclastic activities. They found that both osteoclasts and bone marrow macrophages were much more prevalent when examined using immunohistochemistry with Cluster of differentiation 68 (CD68)^[36].

The results of this study revealed increased infiltration of inflammatory cells in the bone marrow cavities compared with the OA group. This could be explained according to **Rojecky et al. 2008**. They investigated the anti-inflammatory effect of botulinum toxin on carrageenan and capsaicin-induced hind paw tissue inflammation. They found a noticeable inflammatory response that was distinguished by an increase of polymorphonuclear leukocytes in interstitial tissue and between muscle bundles^[37].

Furthermore, **Cayan et al. 2003**, investigated how botulinum toxin type A affected bladder histology and function in a rat model of chemical cystitis. They found improvement in the function of the bladder, but histologically there was not a noticeable improvement as the inflammatory cells (mast cells and leukocytes) were not decreased. They concluded that botulinum neurotoxin had no anti-inflammatory effect^[38].

The combination of Botox and HA for the treatment of induced TMJ-OA was covered for the first time in this study. The results of this research showed that the thickness of articular cartilage

increased insignificantly in comparison to the OA group and significantly compared with the Botox group but was still lower than that of the HA group and the control group. Hypertrophy, clustering, and empty lacunae can all be seen in chondrocytes. Subchondral and temporal bones improved over the Botox group, but not as much as the HA group or control groups. Infiltrated inflammatory cells were lower than in the Botox group but still higher than in the HA group.

Tumor necrosis factor (TNF- α), a potent cytokine, is released in the body in response to inflammation, infection, and injury. TNF- α causes a wide range of cellular and organismal reactions, such as lymphocyte and leukocyte activation and migration, fever, cell proliferation, differentiation, and apoptosis. TNF- α is a “master regulator” of the inflammatory process, according to growing evidence, as it can encourage the production of other inflammatory cytokines^[39]

Tumor necrosis factor receptors (TNFR) 1 and 2 are two cell-membrane receptors that TNF- α uses to exert its biological effects^[40]. **Zhang et al.** demonstrated that TNFR1 plays a crucial role in mediating all phases of inflammatory pain, while TNFR2 has a special role in mediating early-phase inflammatory pain in mice. When TNF- α binds to TNFRs, several pathways are activated, including nuclear factor-kappa B (NF-B), p38 mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK), which causes cell death through necroptosis or apoptosis and results in the production of several important cytokines^[41].

The synovial fluid, cartilage, synovial membrane, and subchondral bone of OA joints all have higher TNF- α levels. TNF- α is known to cause chondrocyte apoptosis in TMJ-OA via activating the mitochondrial and apoptosis receptor pathways. Additionally, chondrocytes have receptors for a number of pro-inflammatory cytokines and chemokines. So, in OA, chondrocytes serve as both the source and the target of proinflammatory cytokines^[42].

In joints, synovium and chondrocytes are responsible for the production of TNF- α , which leads to the release of degrading enzymes such as matrix metalloproteinases (MMP) and collagenase. As a result of the release of these enzymes, bone and cartilage degenerate^[43].

In the current study, the number of immunopositive chondrocytes of articular cartilage in the HA group was significantly lower compared with the OA group, Botox group, and combination group ($p < 0.05$). In the Botox group, the number of positive cells was significantly higher compared with the HA group and combination, and insignificantly higher compared with the OA group.

Under these facts, together with our histologic and immunohistochemical findings, the current study concluded that CFA-induced inflammation was caused by the induction of immunological responses, as shown by an increase in TNF- α levels in the articular cartilage. HA and combination (HA and Botox) can antagonize joint inflammation, as demonstrated by the reversal of the elevated levels of inflammatory cytokines (TNF- α) in the inflamed articular cartilage. Botox alone failed to reduce the level of TNF- α .

TNF- α may be linked to inflammation, neuropathic and inflammatory pain in the TMJ, and deterioration of the TMJ bone and cartilage. As a result, future treatments for TMD may benefit from preventing both TNF- α production and its effects^[44].

CONCLUSION

According to the findings of the current study, intraarticular injection of HMWHA led to a reduction of inflammation and regeneration of cartilage and bone. While intra-articular Botox injections failed to reduce inflammation and promote cartilage and bone regeneration. The first time intra-articular injections of combination (HA and Botox) were studied, we showed a minor improvement in osteoarthritic features.

REFERENCES

1. Monasterio, G., et al., Osteoarthritis of the temporomandibular joint: clinical and imagenological diagnosis, pathogenic role of the immuno-inflammatory response, and immunotherapeutic strategies based on T regulatory lymphocytes, in *Temporomandibular Joint Pathology-Current Approaches and Understanding*. 2018, IntechOpen.
2. de Souza, R.F., et al., Interventions for managing temporomandibular joint osteoarthritis. *Cochrane database of systematic reviews*, 2012(4).
3. Kogan, G., et al., Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology letters*, 2007. 29(1): p. 17-25.
4. Abramoff, B. and F.E. Caldera, Osteoarthritis: pathology, diagnosis, and treatment options. *Medical Clinics*, 2020. 104(2): p. 293-311.
5. Rydell, N. and E.A. Balazs, Effect of intra-articular injection of hyaluronic acid on the clinical symptoms of osteoarthritis and on granulation tissue formation. *Clinical Orthopaedics and Related Research*, 1971. 80: p. 25-32.
6. Guarda-Nardini, L., S. Masiero, and G. Marioni, Conservative treatment of temporomandibular joint osteoarthritis: intra-articular injection of sodium hyaluronate. *Journal of Oral Rehabilitation*, 2005. 32(10): p. 729-734.
7. Guarda-Nardini, L., et al., Treatment of patients with arthrosis of the temporomandibular joint by infiltration of sodium hyaluronate: a preliminary study. *European archives of oto-rhino-laryngology*, 2002. 259(5): p. 279-284.
8. Julovi, S.M., et al., Inhibition of interleukin-1 β -stimulated production of matrix metalloproteinases by hyaluronan via CD44 in human articular cartilage. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, 2004. 50(2): p. 516-525.
9. Elmorsy, S., et al., Chondroprotective effects of high-molecular-weight cross-linked hyaluronic acid in a rabbit knee osteoarthritis model. *Osteoarthritis and cartilage*, 2014. 22(1): p. 121-127.
10. Lajeunesse, D., et al., Hyaluronic acid reverses the abnormal synthetic activity of human osteoarthritic subchondral bone osteoblasts. *Bone*, 2003. 33(4): p. 703-710.
11. Nayyar, P., et al., Botox: broadening the horizon of dentistry. *Journal of clinical and diagnostic research: JCDR*, 2014. 8(12): p. ZE25.
12. Moore, A., General and clinical aspects of treatment with botulinum toxin. *Handbook of botulinum toxin treatment*, 1995: p. 28-53.

13. Yuce, P., et al., Effects of intra-articular resveratrol injections on cartilage destruction and synovial inflammation in experimental temporomandibular joint osteoarthritis. *Journal of Oral and Maxillofacial Surgery*, 2021. 79(2): p. 344. e1-344. e12.
14. Tolba, Y.M., et al., Effect of high molecular weight hyaluronic acid in treatment of osteoarthritic temporomandibular joints of rats. *Archives of Oral Biology*, 2020. 110: p. 104618.
15. Wenger, S., Anesthesia and analgesia in rabbits and rodents. *Journal of exotic pet medicine*, 2012. 21(1): p. 7-16.
16. Arafat, S. and S. Kamel, Biochemical and Histological Evaluation of different intra-articular injections as therapy for Temporomandibular Joint Osteoarthritis in Rats. *Egyptian Dental Journal*, 2021. 67(2): p. 1165-1175.
17. Yaltirik, M., et al., The Effects of intra-Articular Injections of Botulinum Toxin A on Experimentally Temporomandibular Joint Osteoarthritis Models. *Clinical and Experimental Health Sciences*, 2018. 8(1): p. 9-13.
18. Bancroft, J.D. and M. Gamble, *Theory and practice of histological techniques*. 2008: Elsevier health sciences.
19. Chu, C., et al., Localization of tumor necrosis factor α in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. 1991, Wiley Online Library. p. 1125-1132.
20. Bjørnland, T., A. Gjaerum, and A. Møystad, Osteoarthritis of the temporomandibular joint: an evaluation of the effects and complications of corticosteroid injection compared with injection with sodium hyaluronate. *Journal of oral rehabilitation*, 2007. 34(8): p. 583-589.
21. Mahdi, H.J., et al., In vivo anti-arthritis and anti-nociceptive effects of ethanol extract of *Moringa oleifera* leaves on complete Freund's adjuvant (CFA)-induced arthritis in rats. *Integrative medicine research*, 2018. 7(1): p. 85-94.
22. Tuncel, J., et al., Animal models of rheumatoid arthritis (I): pristane-induced arthritis in the rat. *PLoS One*, 2016. 11(5): p. e0155936.
23. Spears, R., et al., Temporal changes in inflammatory mediator concentrations in an adjuvant model of temporomandibular joint inflammation. *Journal of orofacial pain*, 2005. 19(1).
24. El-Hakim, I., I. Abdel-Hamid, and A. Bader, Temporomandibular joint (TMJ) response to intra-articular dexamethasone injection following mechanical arthropathy: a histological study in rats. *International journal of oral and maxillofacial surgery*, 2005. 34(3): p. 305-310.
25. Xu, L., et al., A time-dependent degeneration manner of condyle in rat CFA-induced inflamed TMJ. *American journal of translational research*, 2016. 8(2): p. 556.
26. Soliman, N.B.E., Effect of intra-articular injection of high-molecular-weight versus low-molecular-weight hyaluronic acid on the articular cartilage structure in experimentally induced knee joint osteoarthritis in albino rats. *Egyptian Journal of Histology*, 2012. 35(3): p. 483-495.
27. Chen, G., et al., Anti-arthritis activity of D-carvone against complete Freund's adjuvant-induced arthritis in rats through modulation of inflammatory cytokines. *Korean J. Physiol. Pharmacol*, 2020. 24(6): p. 453-462.
28. Duygu, G., et al., The effects of high molecular weight hyaluronic acid (Hylan GF 20) on experimentally induced temporomandibular joint osteoarthritis: part II. *International journal of oral and maxillofacial surgery*, 2011. 40(12): p. 1406-1413.
29. Li, P., et al., Hylan GF 20 maintains cartilage integrity and decreases osteophyte formation in osteoarthritis through both anabolic and anti-catabolic mechanisms. *Osteoarthritis and cartilage*, 2012. 20(11): p. 1336-1346.
30. KIM, C.-H., et al., Therapeutic effect of hyaluronic acid on experimental osteoarthritis of ovine temporomandibular joint. *Journal of Veterinary Medical Science*, 2001. 63(10): p. 1083-1089.
31. Chakravarti, A., et al., Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption. *Blood, The Journal of the American Society of Hematology*, 2009. 114(8): p. 1633-1644.
32. Lemos, G.A., et al., Effects of high molecular weight hyaluronic acid on induced arthritis of the temporomandibular joint in rats. *Acta histochemica*, 2015. 117(6): p. 566-575.
33. Williams, V.S., Intraarticular hyaluronic acid supplementation in the horse: The role of molecular weight. *Journal of equine veterinary science*, 2007. 27(7): p. 298-303.
34. Mohammed, S.S., Condylar cartilage degradation as response to single dose Botulinum toxin-A injection in masticatory muscles of young rabbits. *Int. J. Adv. Res. Biol. Sci*, 2019. 6(2): p. 94-109.
35. Namazi, H., Botulinum toxin as a novel addition to anti-arthritis armamentarium: An experimental study in rabbits. *International immunopharmacology*, 2006. 6(11): p. 1743-1747.
36. Ali, D., W. Abdelzاهر, and S. Abdel-Hafez, Evaluation of the rivastigmine role against botulinum toxin-A-induced osteoporosis in albino rats: A biochemical, histological,

- and immunohistochemical study. *Human & experimental toxicology*, 2018. 37(12): p. 1323-1335.
37. Bach-Rojecky, L., M. Dominis, and Z. Lacković, Lack of anti-inflammatory effect of botulinum toxin type A in experimental models of inflammation. *Fundamental & clinical pharmacology*, 2008. 22(5): p. 503-509.
38. Cayan, S., et al., Botulinum toxin type A may improve bladder function in a rat chemical cystitis model. *Urological research*, 2003. 30(6): p. 399-404.
39. Hosseinzadeh, A., et al., Apoptosis signaling pathways in osteoarthritis and possible protective role of melatonin. *Journal of pineal research*, 2016. 61(4): p. 411-425.
40. Cabal-Hierro, L. and P.S. Lazo, Signal transduction by tumor necrosis factor receptors. *Cellular signalling*, 2012. 24(6): p. 1297-1305.
41. Zhang, L., et al., TNF-alpha contributes to spinal cord synaptic plasticity and inflammatory pain: distinct role of TNF receptor subtypes 1 and 2. *PAIN®*, 2011. 152(2): p. 419-427.
42. Qin, J., et al., TNF/TNFR signal transduction pathway-mediated anti-apoptosis and anti-inflammatory effects of sodium ferulate on IL-1 β -induced rat osteoarthritis chondrocytes in vitro. *Arthritis research & therapy*, 2012. 14(6): p. 1-12.
43. Sandell, L.J. and T. Aigner, Articular cartilage and changes in arthritis: cell biology of osteoarthritis. *Arthritis Research & Therapy*, 2001. 3(2): p. 1-7.
44. Wang, Y., et al., The Role of TNF- α in the Pathogenesis of Temporomandibular Disorders. *Biological and Pharmaceutical Bulletin*, 2021. 44(12): p. 1801-1809.