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HISTOLOGICAL AND MOLECULAR ASSESSMENT OF ANTI-INFLAMMATORY EFFECT OF DIFFERENT CONCENTRATIONS OF HYALURONIC ACID INJECTED IN INFLAMED RABBIT'S TEMPOROMANDIBULAR JOINT

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KEYWORDS

CRP, Hyaluronic acid, IL-6, IL-1β, TMJ.

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ABSTRACT

Introduction: Several studies indicated that Hyaluronic acid (HA) has anti-inflammatory role through suppressing mediators of inflammation involved in the osteoarthritic disease state. **Aim:** Investigating the anti-inflammatory effect of different concentrations of hyaluronic acid on experimentally induced inflammatory changes in rabbits temporomandibular joint (TMJ). **Material and Methods:** Forty male rabbits were prepared and randomly divided into 5 groups each group formed of 8 rabbits as follow: ***Group (1):*** 8 animals injected with saline only served a negative control group. ***Group (2):*** 8 animals injected with formalin served as positive control. ***Group (3):*** 8 animals injected with 0.1ml of formalin once, followed by 2.5mg/0.5ml /week of HA for three weeks. ***Group (4):*** formed of 8 animals injected with 0.1ml of formalin once, followed by 5mg/0.5ml /week of HA for three weeks. ***Group (5):*** 8 animals injected with 0.1ml of formalin once, followed by 7.5mg/0.5ml /week of HA for three weeks. Eventually blood samples were collected and animals were euthanized. Evaluation in this study included measuring pro inflammatory markers IL-6, IL-1β and CRP by ELISA. Histological evaluation of TMJ tissues to detect changes in the muscles, ligaments, and bones. **Results:** all animals injected with HA showed a significant reduction in levels of IL-6, IL-1β and CRP in addition to improvement in tissue structure. Animals in G4 and G5 showed significantly better results than G3. **Conclusion**: there is a statistically significant anti-inflammatory effect of HA on TMJ at different concentrations.

INTRODUCTION

Hyaluronic acid has a wide range of applications in oral and maxillofacial surgery. These include intra-articular injections of hyaluronic acid for treatment of different conditions such as treating tempromandibular joint osteoarthritis, treating internal derangement and reducing symptoms in patients with disc displacement with and without reduction[(1)]. Moreover, application of hyaluronic acid into extraction site after impaction to reduce postoperative pain and edema and in periodontal therapy [(1,2)].

Hyaluronic acid is a non-branched linear polysaccharide consisted of several units of glucuronic acid disaccharides and Nacetylglucosamine. It is produced by synoviocytes, fibroblasts and chondrocytes

found in the mammals connective tissues. In addition, it represents the main component of synovial fluid and articular cartilage [2].

Many of the biological actions of HA (hyaluronic acid) are dependent on the molecular size of the ligand. Hyaluronic acid synthases (HAS) is responsible for formation of long HA polymers often ranging from lower molecular weight to higher molecular weight HA, as high as 5 × 106 kDa[3]. HA destruction is done by Hyaluronidase-1 (Hyal-1) and hyaluronidase-2 (Hyal-2) enzymes through fragmentation of HA from both ends toward the center of the molecule, which decreases the molecular weight and creates HA oligosaccharide fragments within the synovial fluid. Some researchers has suggested that anti-inflammatory characters are related to long polymer HA, while short HA oligisacharide mediates receptor signaling pathways to initiate pro-inflammatory reactions[4].

Hyaluronic acid has mechanical, metabolic and biological actions. The mechanical role is achieved by minimizes wear of articular surfaces through keeping them lubricated. Also, it plays a pivotal role in nutrition and metabolism of the articular disc and cartilage [5]. Regarding the biological effect, researchers found an imporntat role of Hyaluronic acid in inhibiting inflammatory mediators such as tumor necrosing factor (TNF),interlukin- 1(IL-1), interlukin- 17(IL-17), Prostaglandin E2 (PGE2)and inducing of nitric oxide synthase (iNOS), as well as preventing the extracellular matrix destruction such as matrix metalloproteinases (MMPs) 1, 3, 2, 9 and 13 [6,7].

While the effect of HA usage as viscosupplement is well documented, its anti inflammatory effect is not clear. Moreover, there is no consensus on the concept of molecular weight. So the aim of this study was to investigate the anti-inflammatory effect of different concentrations of HA injected in inflamed rabbits TMJ

MATERIALS AND METHODS

I. Ethical Consideration:

This experimental study was approved from the ethical committee (ERC) Faculty of Dentistry, Suez Canal University with approval number (204/2020).

II. Drugs and chemicals:

1. Hyaluronic acid :ampules hyaluronic acid sodium salt 1.5%(Hyalone 60mg/4ml):Fidia Farmaceutici S.p.A.
2. Formaldehyde 37% solution ,Foremark Performance Chemicals, USA
3. Sodium chloride :0.9% solution, Pharco Pharmaceuticals, EG.
4. Ketamine HCL: 500mg/10ml Dechra Pharmaceuticals, UK.
5. ELISA: Kits:Becton, Dickinson Comapany, Franklin Lakes, NJ.
6. Sodium pentobarbital: 100 mL in 1 VIAL, Virbac AH, Inc., Fort Worth, TX.

Preparation of different concentrations of HA:

- **2.5mg/0.5ml**: the concentration was prepared by adding 4ml of saline to each 1ml of original concentration.
- **5mg/0.5ml**: the concentration was prepared by adding 2ml of saline to each 1ml of original concentration.
- **7.5mg/0.5ml**: the original concentration of packaging.

III. Experimental design

Forty New Zealand white rabbits (male, on average 18 weeks old and 3.5kg) were used in this study. They purchased from the Agriculture

collage in Suez Canal University. Rabbits were housed in clean well-ventilated stainless steel cages, and temperature at 25±3°C throughout the experiment and left one week for acclimatization. The Experiment conducted in the Pharmacology Department, lab animal at Faculty of Medicine, Suez Canal University.

IV. Experimental Procedures:

IV.1. Arthritis induction: One week before Hyaluronic acid administration, all animals (except the negative control group), were received intra- articular injection with formalin for arthritis induction. Original package came with 7.5mg/0.5ml then it was diluted by adding sterile saline to reach wanted concentrations.

IV.2. Treatment protocol: Hyaluronic acid were injected intra-articularly in temporomandibular joints at concentrations 2.5mg/0.5 ml, 5mg/0.5 ml and 7.5 /0.5mg respectively once a week for 3 weeks.

Steps of HA application

- Rabbits were anesthetized with an intramuscular injection of ketamine (20 mg /kg) .
- Subjected to bilaterally TMJ injection of formalin (1.5% concentration); using 30-gauge needle introduced into the TMJ. The needle were inserted in the posteroinferior border of the zygomatic arch and advance in an anterior direction until it contact the posterolateral aspect of the condyle at a depth of approx. 3mm[8].

V. Animal Grouping:

Animals were divided into 5 groups and each group formed of 8 rabbits :

Group (1): Negative control group, formed of 8 animals and injected with saline only.

Group (2): Positive control group (with induced arthritis), formed of 8 animals and injected with formalin only.

Group (3): 8 animals injected with 0.1ml of formalin. After arthritis induction treated by 2.5mg/0.5ml of HA injection for two weeks .

Group (4): 8 animals injected with 0.1ml of formalin. After arthritis induction treated by 5mg/0.5ml of HA injection for two weeks .

Group (5): 8 animals injected with 0.1ml of formalin. After arthritis induction treated by 7.5mg/0.5ml of HA injection for two weeks.

Samples collection:

After five weeks from the 1st injection the blood samples were taken from all rabbits in all groups then they were euthanized by overdose of Sodium pentobarbital to harvest tissue samples [9] .

VI- Evaluation methods:

VI- 1. Inflammatory markers evaluation :

Blood samples were collected for detecting anti-inflammatory markers by measuring serum C- reactive protein (CRP), IL-6 and IL-1β by ELISA diagnostic kit.

VI- 2. Histological evaluation:

For detection of any pathological changes, TMJ tissues were separated and fixed in a 10% phosphate-buffered paraformaldehyde solution. Tissues were dehydrated and decalcified using 8 % trichloroacetic acid for 4 weeks. Paraffin-embedded specimens were sagittally cut in serial sections, embedded in paraffin and stained with hematoxylin and eosin (H&E). Then specimens were examined under a light microscope.

Statistical Analysis

Results were collected and expressed as Mean ± SD. Results will be analyzed using the statistical package for the social sciences, version 19 (SPSS Software, SPSS Inc., Chicago, USA). Results were expressed in the form of p-values that were differentiated into :

- Non significant when p- value >0.05.

- Significant when p- value <0.05.

RESULTS

I. Histological evaluation

Group 1 (Negative control):

Histological evaluation of the rabbit's TMJ of control group showed the normal histological picture of different components of the TMJ articulating surfaces and articular disc between them. ***The condyle*** the outer surface was appeared smooth and regular covered with fibrocartilage tissue. The fibrocartilage layer consisted of clearly identified zones arranged as fibrous, proliferative, mature, hypertrophic cartilaginous zones. The deep part of calcified cartilage showed necrotic hypertrophic chondrocytes surrounded by a calcified matrix. The subchondral bone was formed of cancellous type of lamellar bone. ***The TMJ disc*** which present between the articulating surface, in the form of biconcave disc formed of wavy, thick collagen fibers and chondroblasts scattered in between, where the thickness increased towards the capsular insertion. ***Temporal bone***: covered by fibrocartilaginous layer (**Figure 1. A:C).**

Group 2 (Positive control): Histological evaluation of the group 2 rabbit's TMJ received formalin injection for induced arthritis revealed several histopathological changes in all different TMJ parts. ***The condylar cartilage*** showed signs of erosion, reduction in thickness in addition to loss of organization of the cartilaginous zones. Chondrocytes showed clusters aggregations. Some areas showed loss of chondrocytes in addition to focal calcification. The outer surface appeared irregular with increase in the fibrous cover in other areas. ***The articular disc*** showed tearing in some parts with loss of its typical biconcave form. Increased density of collagen, less cellularity, with randomly arranged hypertrophic chondrocytes. The normal appearance of the temporal bone get altered, where the cartilaginous coverage showed reduction in thickness accompanied with increase in the fibrous layer in some samples. The synovial membrane showed hypertrophy towards the articular cavity, in addition to inflammatory cells infiltrate (**Figure 1.D:I**).

Group 3 (2.5mg/0.5ml): Histological evaluation of the group 3 rabbit's TMJ received formalin injection for induced arthritis and treated with HA of 2.5 concentrations revealed some degree of improvement when compared to the group 2.

The condylar cartilage showed increase of its thickness, increased number of chondrocytes especially hypertrophic zone, however chondrocytes still showed disorganized zones in addition to some cluster aggregation still present. Temporal bone and articular disc showed almost normal morphology. Moreover there were not inflammatory (**Figure 2. A:C**).

Group 4 (5mg/0.5ml): Histological evaluation of the group 4 rabbit's TMJ received formalin injection for induced arthritis and treated with high wight Hyaluronic acid of 5 concentration showed improvement in the histological structure when compared to the group 2, where the condylar cartilage, temporal bone and articular disc showed almost normal histological appearance without any inflammatory cells (**Figure 2. D: F**)

Group 5 (HA 7mg/0.5ml): Histological evaluation of the group 5 rabbit's TMJ received formalin injection for induced arthritis and treated with HA of 7 concentration revealed signs of improvement when compared to the group 2, where the condylar cartilage showed increase of its thickness, however chondrocytes showed less organized zones appearance. Temporal bone and articular disc showed almost normal morphology and there were not any inflammatory cells (**Figure 2. G:I)**

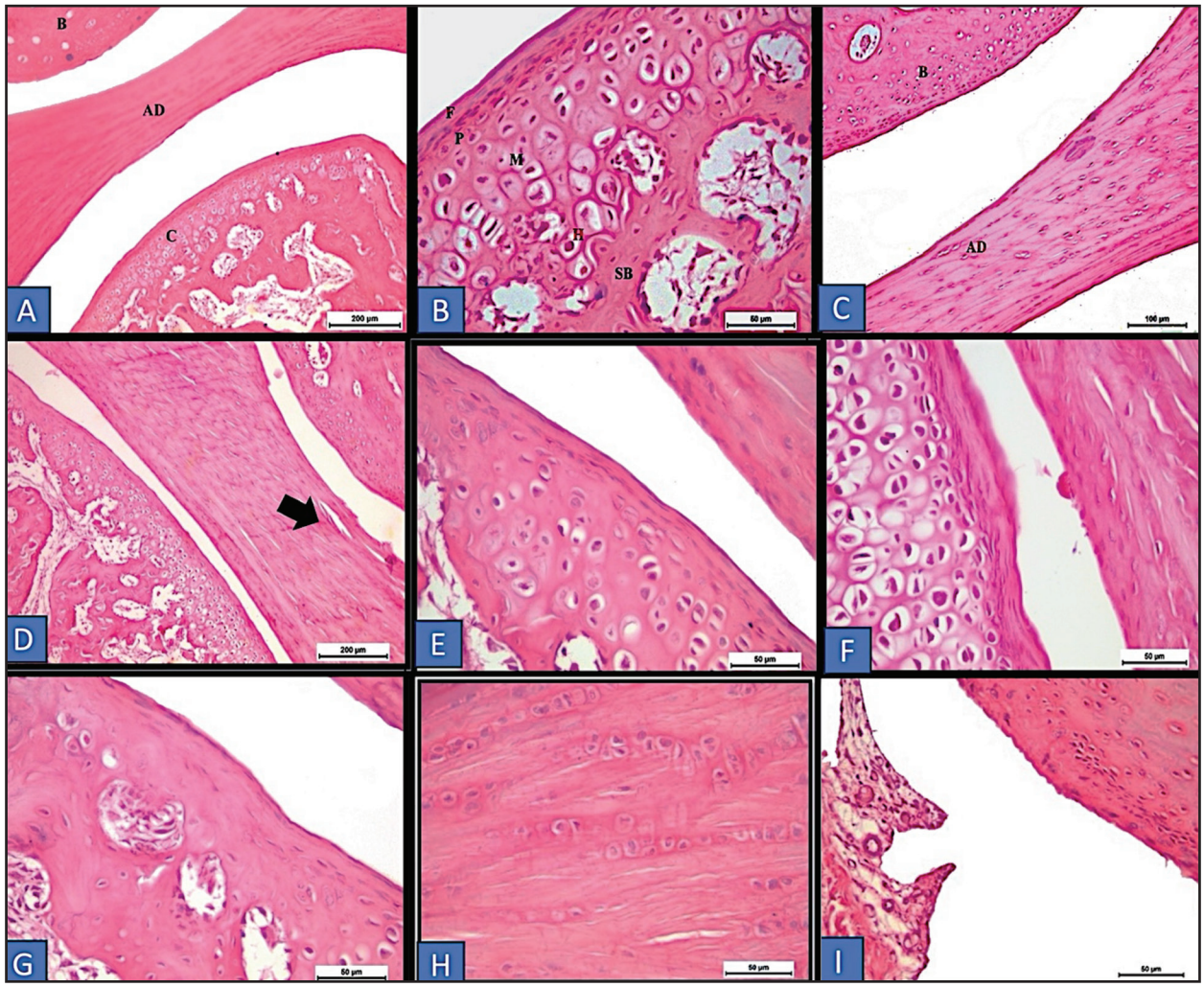

Fig. (1) **A photomicrograph of rabbit's TMJ of negative control (A:C) and positive control (with induced arthritis) groups (D:I) A.**: Negative control group showing condylar cartilage (G), biconcave articular disc (AD) and temporal bone (B) separated by Temporodiscal space and condylodiscal space. **B.**: showing well organized zones of condylar cartilage arranged from outside as fibrous (F), proliferative (p), mature (M), hypertrophic(H) and underlying subchondral bone(SB). **C.**: normal appearance of smooth and regular temporal bone surface covered by fibrocartilaginous layer and articular disc. **D.** showing loss of biconcave appearance of the articular disc, in addition to some clefts (arrow). **E.** showing reduction in the thickness of the condylar cartilage accompanied with reduction of chondrocyte. **F.** showing cluster aggregation of chondrocytes in the condylar cartilage in addition to surface irregularities on the condylar cartilage outer surface. **G.** showing focal calcification and loss of chondrocytes of the condylar cartilage. **H.** showing hypertrophied chondrocytes with disorganized arrangement in the articular disc. **I.** showing temporal bone with surface irregularities in addition to hypertrophic synovial membrane with inflammatory cells.

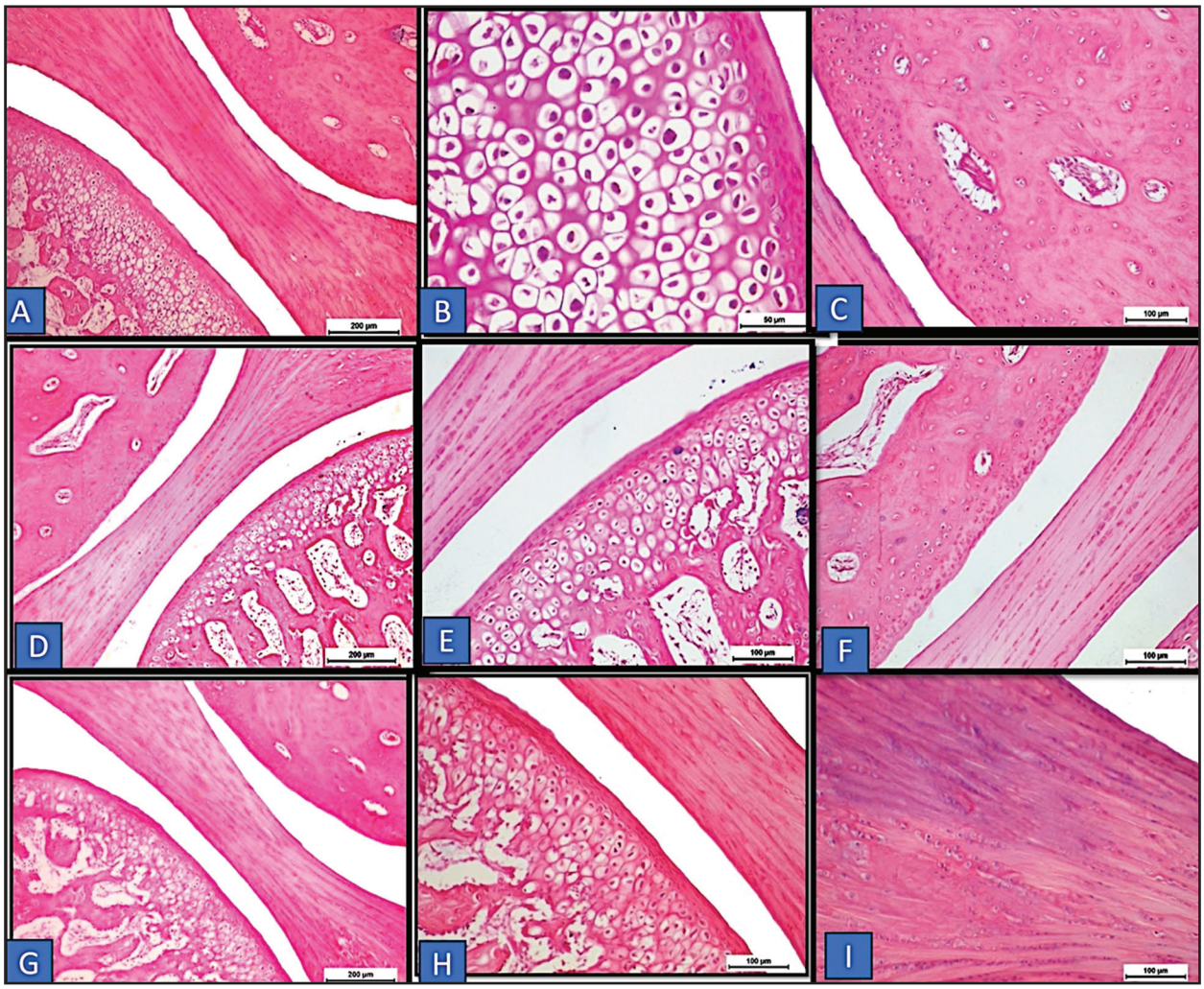

Fig. (2) A photomicrograph showing of Sagittal section in rabbit's TMJ of group 3 ,4 &5; **Group 3**, (**A:C**); **A.** Showing improvement in the overall appearance of the articulating surfaces and the articular disc. **B.** showing an increase in the condylar cartilage thickness, mainly the hypertrophic zone. **C.** Showing temporal bone appeared with smooth regular surface and normal thickness of its fibrocartilaginous cover. **Group 4**, **(D:F). D**. Showing normal appearance of the articulating surfaces and the articular disc. **E.**: Normal appearance of condylar cartilage with well-organized zones arrangement. **F.** Almost normal appearance of the temporal bone. **Group 5**, **(G:I).** group 5: **G.** almost normal structure of the articulating surfaces and the articular disc. **H**. Showing increase in the thickness of the condylar cartilage. **I.** Showing the articular disc, chondrocytes arranged in rows in between the collagen fibers as in group 1.

II-Laboratory Investigations :

1. CRP levels

Table 1 showed that there was a statistically significant difference between the studied groups regarding CRP (P< 0.001).

Table (1) *Comparison of CRP levels between the studied groups:*

	GI (Saline)	GII (Formalin)	GIII (2.5mgml)	GIV (5mgml)	GV (7.5mgml)	P
CRP	54.50 ±2.263	118.50 ±8.202	75.97 ±2.486	64.77 ±3.915	53.50 ±2.025	**< 0.001**

Data is expressed as mean and standard deviation. P is significant when < 0.05.

2. CRP between the studied groups

In table 2, the post hoc analysis showed that there was a statistically significant difference regarding the CRP between normal group and formalin groups (p< 0.001), normal group and group III (p< 0.001), group IV and formalin (p< 0.001), group V and formalin (p< 0.001) while there was not a significant difference between group III and formalin (p =.974), saline group and GV (p= 0.998), saline group and GIV (p= 0.051).

Table (2) *Post hoc analysis of CRP between the studied groups:*

CRP	
GI-GII	**< 0.001**
GI-GIII	**< 0.001**
GI-GIV	0.051
GI-GV	0.998
GIII-GII	0.974
GIV-GII	**< 0.001**
GV-GII	**< 0.001**
P is significant when < 0.05.	

3. IL- 1β levels

Table 3 showed that there was a statistically significant difference between the studied groups regarding IL- 1β (P< 0.001).

Table (3) *Comparison of IL- 1β levels between the studied groups:*

	GI (Saline)	GII (Formalin)	GIII (2.5mgml)	GIV (5mg/ml)	GV (7.5mgml)	P
IL- 1β	113.90 ± 1.838	230.30 ± 17.112	180.30 ± 13.852	165.45 ± 18.166	121.30 ± 21.771	**< 0.001**

Data is expressed as mean and standard deviation. P is significant when < 0.05.

4. IL-1B between the studied groups

In table 4, the post hoc analysis showed that there was a statistically significant difference regarding the IL-1B between normal group and formalin groups (p< 0.001), normal group and GIII (p= 0.007), normal and group IV (p= 0.035), group III and formalin (p= 0.027), group IV and formalin (p= 0.008), group V and formalin (p< 0.001), while there was not a significant difference between group I and group V (p= 0.986).

Table (4) *Post hoc analysis of IL-1B between the studied groups:*

IL- 1B	
GI-GII	**< 0.001**
GI-GIII	**0.007**
GI-GIV	**0.035**
GI-GV	**0.986**
GIII-GII	**0.027**
GIV-GII	**0.008**
GV-GII	**< 0.001**
P is significant when < 0.05.	

5. IL- 6 levels

Table 5 showed that there was a statistically significant difference between the studied groups regarding IL- 6 (P< 0.001).

Table (5) *Comparison of IL-6 levels between the studied groups:*

	GI (Saline)	GII (Formalin)	GIII (2.5mg/ml)	GIV (5mg/ml)	GV (7.5mg/ml)	P
IL-6	220.65 ± 4.455	412.20 ± 4.384	319.55 ± 19.231	306.63 ± 37.532	240.30 ± 21.999	**< 0.001**

Data is expressed as mean and standard deviation. P is significant when < 0.05.

6. IL-6 between the studied groups

In table 6, the post hoc analysis showed that there was a statistically significant difference regarding the IL-6 between saline group and formalin groups (p< 0.001), saline group and group III (p= 0.006), group III and formalin (p= 0.009), group IV and formalin (p= 0.003), group V and formalin (p< 0.001), while there was not a significant difference between group V and group I (p= 0.857). saline group and group IV (p= 0.998).

Table (6) *Post hoc analysis of IL-6 between the studied groups:*

	IL-6
GI-GII	**< 0.001**
GI-GIII	**0.006**
GI-GIV	0.988
GI-GV	0.857
GIII-GII	**0.009**
GIV-GII	**0.003**
GV-GII	**< 0.001**
P is significant when < 0.05	

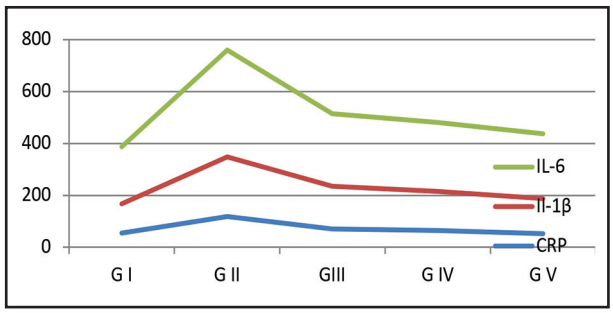

Fig. (3) Showing the effect of different HA concentrations on IL-6 ,CRP and IL-1β

DISCUSSION

One of the major pathologies affecting the temporomandibular joint (TMJ) is Osteoarthritis. The usage of intra-articular hyaluronic acid (HA) is scientifically approved in treating TMJD (Tempro Mandibular Joint Disorders) through reducing inflammatory mediators and acting as viscosupplement [10].

When studying inflammatory disorders of the TMJ. Thereare similarities of rabbits TMJ to humans TMJ in terms of its anatomy and movements, mainly the laterality that gives it a mobility not found in other animals [11].

Because of there is no consensus among researchers about concentrations and repetition of HA doses, different concentrations have been used in this research and repeated 3 times after osteoarthritis induction to investigate the most effective dose [12] .

In this study, the intra-articular injection of formalin in G2, caused substantial morphological changes in the different TMJ Components The condylar cartilage, chondrocytes, articular disc and temporal bone get altered.

On the other hand, animals injected with different concentrations of HA showed minor morphological changes compared to those of induction group. Showing a significant effect of different concentrations of HA in the reduction of inflammatory cells, synovial membrane hyperplasia, articular cartilage destruction and bone abnormalities in the TMJ. While the effects on G3 (2.5mg/0.5ml) were minor, G4 (5mg/0.5ml and G5 (7.5mg/0.5ml) TMJ tissues showed better healing .In G4 and G5 The condylar cartilage showed increase of its thickness, temporal bone and articular disc showed almost normal morphology .

The results obtained here were suggested to resulted from the anti inflammatory effects of HA, as reported in the literature. As well as to, collagen fibers degradation enzymes such as MMPs, this explain the anti inflammatory effects of HA [13].

Also, in the present study temporal bone and articular disc showed almost normal morphology in HA treated groups (G4 &G5) which in agreement with other studies who revealed that HA stimulate bone healing through accelerating the three phases of healing; inflammation, proliferation and migration mesenchymal cells and they confirmed that HA needs an osteoconductive scaffold to be effective. Other study illustrated that the HA helped in bone formation by enhancing osteogenesis and accelerating bone healing process through cell adhesion promotion and differentiation of osteoblast o [14,15].

Another study which support the present results stated that HA mixtures seemed to have a role in cartilage protection by inhibiting proteoglycan loss and be promoting type II proteoglycan loss[16].

Regarding the cytokine level, the current study reported significantly high levels of IL-6, IL-1B and C- reactive protein in the control group. This corresponds to a previous study(16) which reported that IL-1B and Il-6 and C- reactive protein have a major role in the pathogenesis of the degenerative changes in TMJ OA. Also, there was a significant reduction in Inflammatory mediators in all treated groups.

In the present study different concentrations of HA drop IL-6 & IL-1β concentration in G3,G4 and G5. There was a significant difference between G3 &G5 group. On the other hand, there was not a significant difference between G4&G5.

These results were in accordance with other researchers who reported that HA has anti-inflammatory effects. HA combined with lavage reduce cytokines levels which can explain the anti-inflamatory effect of HA. IL-1β is the key mediator in anti-inflammatory effects of HA and is regulated through HA-CD44 binding. In vitro studies have shown that by blocking the effect of IL-1b ,HA raises PG levels and prevent further cartilage damage by forming a protective layer around it.

Also, this study results were in agreement with *Gurung et al*[18] who stated that intra-articular HA treatment is effective as anti-inflammatory because its ability to downregulate MMP.

Regarding the IL-1β level, the current study reported significantly high levels of IL-1β in the control group. This corresponds to the previous studies which reported that IL-1β has a major role in the pathogenesis of the degenerative changes in TMJ OA[19]. These results support the research results of *Zhang et al*[20] who demonstrated that fibrosis ,a shift to chronic inflammation and subsequent degeneration of TMJ are caused by IL-1B .

This is consistent with the studies performed by *Gulen et al*[21], who reported that healthy joints synovial fluid lack inflammatory mediators and arthrocentesis for unhealthy joints led to drop in the levels of inflammatory mediators. Our results were also found to be in agreement with *Sezgin et al* [22] found that IL-6 levels decreased abruptly after injecting knee joints with HA.

IL-6 plays a complex and dual role in RANKL-induced osteoclastogenesis. Through suppressing the proliferation of osteoclast progenitors and downregulating RANKL expression on mature osteoclast IL-6 inhibit bone resorption[23]. On the other hand, high levels of IL-6 induce bone resorption by binding to soluble IL-6R and directly induce RANKL expression on osteoblasts and on synovial fibroblasts[24]. It is reported that when there were bony changes in the TMJ condyle, the levels

of IL-6 and interleukin-11 (IL-11) in synovial fluid increased, suggesting that IL-6 and IL-11 might play a role in the pathogenesis of TMD[25]. In GS-treated rats, expression of RANKL in the joints could be inhibited by reducing IL-6 levels. Besides, high concentrations of IL-6 were detected during early OA[21].

IL-6 has been related to TMJ damage. It has been suggested that the mandibular condylar cartilage damage of rats in inflammatory conditions is affected by IL-1β induced by COX-2/PGE2.

There was a significant drop in CRP levels in groups when compared with the control group. In addition to that G3,G4amd G5 reached similar levels to normal.

Pervious studies showed contradictory results which caused uncertainty about the association between CRP expression and OA progression for a long time [26]. In some studies patients diagnosed with RA showed higher levels of CRP which correlated to resorbed condyles in the TMJ, seen on CT and low jaw opening capacity [27]. Some researchers linked these results to the protective role of CRP during the early stage of RA while exaggerate the tissue response during late stages [28]. In addition, genetic variation is a factor progression of RA[29]. Thus, serum CRP levels are affected by the stage of inflammation and genetic variation[30].

CONCLUSION

According to the findings of the current study intraarticular injection of HA at 5mg/.05ml and 7.5mg/.05m concentration lead to a significant reduction of inflammation through reduction of inflammatory markers and improvement of histological picture of rabbit's TMJ.

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