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# Ameliorative Effect of Copper Albumin Complex on Proteoglycan in Mono-iodoacetat Induced Osteoarthritis Rat Model

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## Abstract

Osteoarthritis (OA) is a degenerative disorder involving the joint, including cartilage and synovial fluids. Recent studies have sought to find curative therapeutics to decrease the adverse effects of OA and relieve associated pain. Globally, knee osteoarthritis (KOA) is the most common type of arthritis. The present study aimed to evaluate the anti-inflammatory effect copper albumen complex (cu-albumin complex) for the treatment of mono-iodoacetate (MIA)-induced KOA in Albino rats. A total of 50 adult male albino rats were involved and divided as follows; 10 rats were kept normal as negative control; 20 arthritic rats were kept untreated (positive control), and 20 arthritic rats were treated with cu-albumin complex orally for a month. Treated and untreated arthritic rats were divided equally (10 rats each) into mild and severe groups according to the severity of signs. The intra-articular injection of MIA in the right knee joint was used for induction of osteoarthritis. Using Mankin grading score, the results demonstrated that the treated groups had a better histological appearance than the control positive group. Additionally, except for a few shrunken chondrocytes, the mildly treated group showed less degenerative alterations and appeared virtually normal. While the severe treated group showed increased cellularity with decreased degenerated chondrocytes. It concluded that balanced copper consumption has a positive impact on the prevention and treatment of KOA.

**Keywords:** Aggrcan, Copper, Knee, MIA, Osteoarthritis, Proteoglycan.

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Competing interest: The authors have declared that no competing interest exists.

## Introduction

Osteoarthritis (OA) is a degenerative disease that affects the joints and its surrounding tissues in a huge number of individuals worldwide, with the highest frequency of all forms of arthritis, resulting in significant morbidity and disability owing to pain. (Lim et al., 2012; Neogi, 2013).

Pathological changes in cartilage, bone, synovium, ligament, muscle, and periarticular fat characterize OA, resulting in loss of joint function, pain, stiffness, functional restrictions, and the loss of valued activities such as walking for exercise. KOA is a prevalent degenerative condition that causes impairment in the elderly (Kan et al., 2019; Katz et al., 2021).

Age, sex, and heredity, as well as a number of modifiable risk factors like physical activity, obesity, and smoking, are all linked to the onset and progression of OA (Felson et al., 2000; Reijman et al., 2007). OA is presently diagnosed using the following criteria: clinical symptoms as pain and loss of function, and radiographic criteria which include joint space width (Heidari, 2011; Nishimura et al., 2011).

Due to radiography's limitations (e.g., technical difficulties, precision, and sensitivity), researchers are looking at alternative parameters for monitoring OA that may be used as biomarkers in drug development (Heidari, 2011; Nishimura et al., 2011).

Clinically, OA is defined by morphological and/or physiological changes in the joint, such as Small joint space, subchondral sclerosis, subchondral cysts, local cartilage degradation, hypertrophic bony reactions, and the production of osteophytes at joint boundaries are all symptoms of osteoarthritis as diagnosed on radiographs (Bijlsma et al., 2011). Although OA affects all joint tissues, the most noticeable change is articular cartilage erosion, which is followed by joint disorder (Eid et al., 2006; Wang et al., 2011).

The essential elements of the extracellular matrix (ECM) in the hyaline cartilage is principally include, water (up to 80% of its dry weight), collagen, and proteoglycans (PG), as well as non-collagenous proteins and trace amounts of glycoproteins. Aggrecan and type II collagen account for the majority of proteins in the articular cartilage ECM, and collagen-binding proteins combine them (Alford and Cole, 2005; Sophia Fox et al., 2009). With fewer levels of non-collagenous proteins and smaller proteoglycans, these two constitute the principal load-bearing macromolecules inside articular cartilage. The compressive and tensile strength of cartilaginous tissue is provided by the interaction of strongly negatively charged cartilage proteoglycans with type II collagen (Chen et al., 2006; Sophia Fox et al., 2009).

Aggrecan, which has over 100 chondroitin sulphate and keratin sulphate chains, is the most prevalent proteoglycan by weight. Aggrecan is found in the interfibrillar region of the cartilage ECM, where it interacts with hyaluronan (HA) via link proteins to form enormous proteoglycan aggregates. It gives cartilage its osmotic qualities, which are necessary for it to withstand compressive loads (Frankle, 1991; Sophia Fox et al., 2009).

The reduction of aggrecan is one of the first signs of OA. Aggrecanase-1 and -2, which are known to cut aggrecan molecules at five places, are assumed to be critical to this degradative process via ADAMTS (A Disintegrin and

Metalloproteinase with Thrombospondin motifs). Aggrecan fragments are commonly detected in synovial fluids from OA joints and inflammatory joint disease, suggesting that aggrecanases may play a role in these joint conditions (Maroudas et al., 1998; Verma and Dalal, 2011; Roughley and Mort, 2014) .

Despite numerous medication treatment options being investigated, the therapeutic impact remains poor because of a lack of long-term circulation and focused delivery capabilities (Xue et al., 2022). In cases of extremely painful OAs or severely decreased joint function, surgery, such as joint replacement, is the only option (Little et al., 2005).

Copper is an important metalloelement that is required for regular human metabolism. It cannot be manufactured in the body, thus it must be consumed and absorbed daily (Sorenson, 1987). Copper complex therapy has been reported to possess anti-inflammatory, analgesic, healing promoter, antipyretic, anticonvulsant, antibacterial, anticancer, antiasthmatic, radioprotectant, angiogenic, anti-carcinogenetic, antiapoptic, and antimutagenic properties (McAuslan and Reilly, 1980; Hu, 1998; Sriram and Lonchyna, 2009; Wangila et al., 2006; El-Badawi et al., 2015). The current study designed to evaluate the anti-inflammatory effect of Cu-albumin complex in the treatment of induced MIA-KOA in albino rats.

## Materials and methods

### Ethical approval:

All experiment was established according to the ethical research committee of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt (Approval No. VM-2022-0032).

### Drugs and chemicals:

cu-albumin complex was provided by Prof. Dr. Ahmed Yassein Nassar, Professor of Biochemistry, Faculty of Medicine, Assiut University, Assiut, Egypt as patent cooperation treaty (PCT) in the international bureau of world intellectual property organization (WIPO), Geneva, Switzerland/ World Organization (WO) 2008 / 028497; Mono-iodoacetate (MIA) was obtained from Sigma Aldrich (St. Louis, MO, USA); Rat proteoglycan Eliza kit was obtained from SinoGeneclon CO., ltd. (Hangzhou, China).

### Animals:

A total of 50 adult male albino rats (3 months old; 130 ± 150g) were purchased from animal house of the Faculty of Medicine, Assiut University, Assiut. Rats were housed individually in plastic cages in a ventilated room with controlled temperature, humidity, and a 12-hour/12-hour light-dark cycle and were fed standard chow with free access of the water. Animals were left to adapt for 10 days and fasted overnight before dosing. Experimental osteoarthritis was induced in rats by intra-articular injection of MIA (3 mg in 50 µl sterile saline) (Guingamp et al., 1997; Udo et al., 2016; Xu et al., 2020) in the right knee joint. Under ethyl acetate anesthesia, the right knee was prepared aseptically by removal of hair using shaver and the area was disinfected by 70% alcohol followed by creation of an incision at the center of the knee to expose the patellar ligament. The right leg was flexed 90˚ at the knee then the injection was made into the region below the patella after locating the patellar ligament (Takahashi et al., 2018).

### Experimental groups:

A week after induction of osteoarthritis, rats were randomly assigned into five groups, 10 rats each and were assigned as follow:

**A- Control group (negative control):**

Rats housed under normal healthy conditions without any osteoarthritis induction.

**B- Non-treated group (positive control, total 20 rats):**

After osteoarthritis induction in the right knee joint, this group left without treatment. Rats were divided according to the severity of signs (pain, swelling and lameness) into a) mild and b) severe not treated groups (10 rats each).

**C- Treated group:**

20 rats, then animals were divided into mild and severe treated groups (10 rats each) according to the severity of signs. After suspension of 817µg/kg copper albumin complex in water, rats were treated orally for a month at a dose of 1 ml/kg B.W daily.

**Animal assessment and Samples collection:**

The swelling of the right knee joint was measured once weekly using a digital caliber. The accelerating rotarod device was used to test joint mobility, motor function, and pain once a week (Ugo Basile, Varese, Italy, Model 7750) according to Vonsy et al., (2009).

**Histopathologic examination of the knee joint (right femorotibial joint):**

After euthanization by cervical decapitation, right femorotibial joint from all groups were cut, washed with saline solution and kept in ethylene diamin tetraacetic acid (EDTA) solution (10%) for decalcification for about one week. Decalcified joints then fixed in 4% neutral buffered formalin (NBF) for 48 hrs. For histopathological examination, the tissue samples were cut into 5 µm thick by Microtome and stained with Hematoxylin and Eosin (H&E) stain, Crossman's trichrome stain (Suvarna et al., 2018). Tissue sections were examined under the light microscope which was supported with digital camera and scored according to modified Mankin score (McNulty et al., 2012; Cui et al., 2015).

**Immunohistochemistry:**

For antigen retrieval, hyaluronidase enzyme (8 mg/mL) was used for 120 min at 37°C and block endogenous peroxidase activity with use of 3% hydrogen peroxide in absolute ethanol for 10 min. The anti-aggrecan primary antibody (Abcam, England) with concentration of 1:50 was added at 4°C for 24 h. The secondary antibody was conjugated with horse radish peroxidase (Abcam) for 60 min, followed by diaminobenzidine (DAB) (DakoCytomation, Denmark), and stained by H&E. The samples were examined using a light microscope.

**Statistical analysis:**

The data were statistically analyzed using the SPSS statistics software version 17.0. (SPSS Chicago, IL, USA). The results were presented as means with ± standard deviations. For multiple intergroup comparisons, one-way analysis of variance was used to assess quantitative differences between values, followed by Turkey tests. A paired sample t-test was used to compare two dependent groups. A statistically significant P-value of $<0.05$ was considered.

**Result**

**H & E staining:**

The normal histological appearance of the articular cartilage was seen in H&E-stained sections of the control group's knee joint. The articular cartilage had a smooth, undamaged surface and typical chondrocytes that were well organized. The chondrocytes were seen in both non-calcified and calcified cartilage, separated by a distinct intact tidemark that showed as a basophilic line. In the non-calcified

region, the chondrocytes were divided into three zones: superficial (tangential), transitional (middle), and radial (deep). Small flat chondrocytes were organized parallel to the articular surface in the superficial zone. Round, oval, or triangular chondrocytes were organized in columns perpendicular to the surface in the transitional and radial zones. The chondrocytes had pale basophilic cytoplasm with central spherical nuclei and were found singly or in groups within their lacunae, producing cell nests. In its lacunae, the calcified zone comprised scattered rounded chondrocytes (Fig. 1).

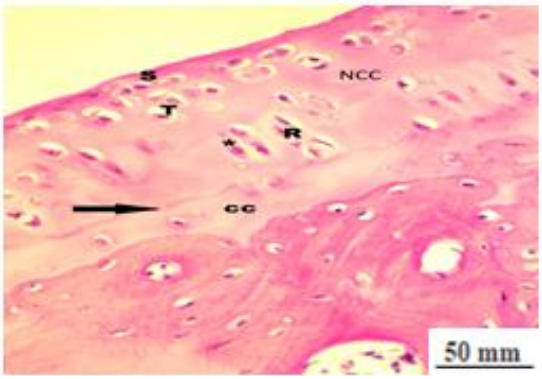


**Fig. 1** Hematoxylin and eosin (H&E)–stained sections of the control group's knee joint show articular cartilage with a smooth intact surface and well-organized chondrocytes in non-calcified (NCC) and calcified (CC) regions of cartilage, with a distinct intact tidemark (arrows) in between. The chondrocytes of the articular cartilage's non-calcified region (NCC) are divided into three zones: superficial (S), transitional (T), and radial (R). Small flat chondrocytes can be found in the superficial zone. Columns of rounded, oval, or triangular chondrocytes (*) can be found in the transitional and radial zones. X400.

In contrast to the control group, slices from the mild osteoarthritis group displayed several histological alterations. The intensity of these modifications varied. The following were the modifications in articular cartilage: disruption of chondrocyte parallel arrangement, irregular notched surface, apparent reduction in cartilage thickness, some chondrocytes shrunken with pyknotic nuclei, disorganised and low in number, chondrocyte loss in some regions, tidemark was faint and irregular (Fig. 2A).

Severe osteoarthritis showed marked degeneration of the articular cartilage. Disarranged and degenerated empty chondrocytes with no visible tidemark were observed (Fig. 2B). In contrast to the osteoarthritis group, samples from the treated groups had a better histological appearance. Except for a few shrunken chondrocytes, the mild treated group showed less degenerative alterations and appeared virtually normal (Fig. 2C). The severe treated group showed increased cellularity with decreased degenerated chondrocytes with faint tidemarks (Fig. 2D).

The articular cartilage in the control group was well stained with aggrecan with no apparent loss of staining intensity, reflecting the normal proteoglycan content of the matrix (Fig. 3). On the other hand, the articular cartilage of the mild osteoarthritis group showed a reduction of aggrecan immunostaining intensity in the entire articular cartilage, reflecting a decrease of the proteoglycan content of the matrix (Fig. 4A).

In the severe osteoarthritis group, a marked decrease in immunostaining was observed (Fig.4B). However, in the mild osteoarthritis treated group, the reduction in aggrecan immunostaining intensity was less pronounced (Fig. 4C). In the severe osteoarthritis treated group, an increase in aggrecan immunostaining was more observed (Fig. 4D).

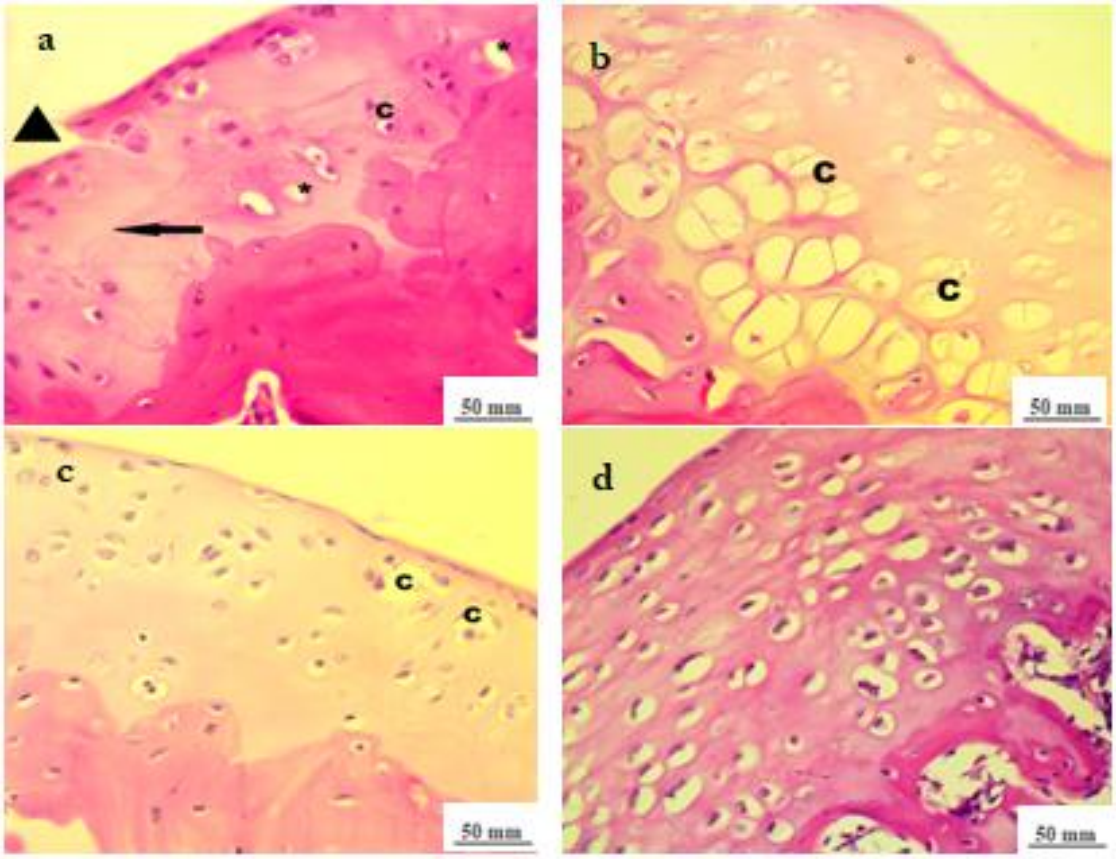


**Fig. 2. A)** Hematoxylin and eosin (H&E)- stained sections of mild osteoarthritis group showing disruption of the parallel arrangement of chondrocytes, irregular notched surface (arrowhead), the apparent reduction in thickness of cartilage, some chondrocytes (C) appear shrunken with pyknotic nuclei, disorganized and few, loss of chondrocytes in some areas (*) and tidemarks are irregular (arrow). X400. **B)** Hematoxylin and eosin (H&E) - stained sections of severe osteoarthritis group showing marked articular cartilage degeneration. Disarranged and degenerated empty chondrocytes (C) with no visible tidemark is observed. X400. **C)** Hematoxylin and eosin (H&E) - stained sections of the mild treated group showing fewer degenerative changes, which appear nearly normal except for few degenerated chondrocytes (C) and invisible tidemark. X400. **D)** Hematoxylin and eosin (H&E) - stained sections of the severe treated group showing increased cellularity with decrease degenerated chondrocytes and invisible tidemarks. X400**.**

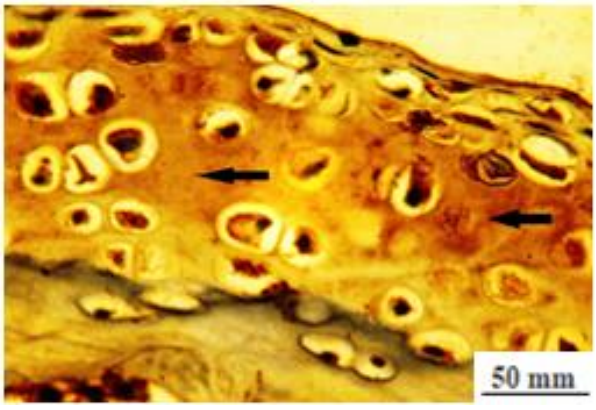


**Fig. 3.** Immunohistochemistry of aggrecan in the articular cartilage of the control group showing that it was well stained with aggrecan with no apparent loss of staining intensity (*). X1000.

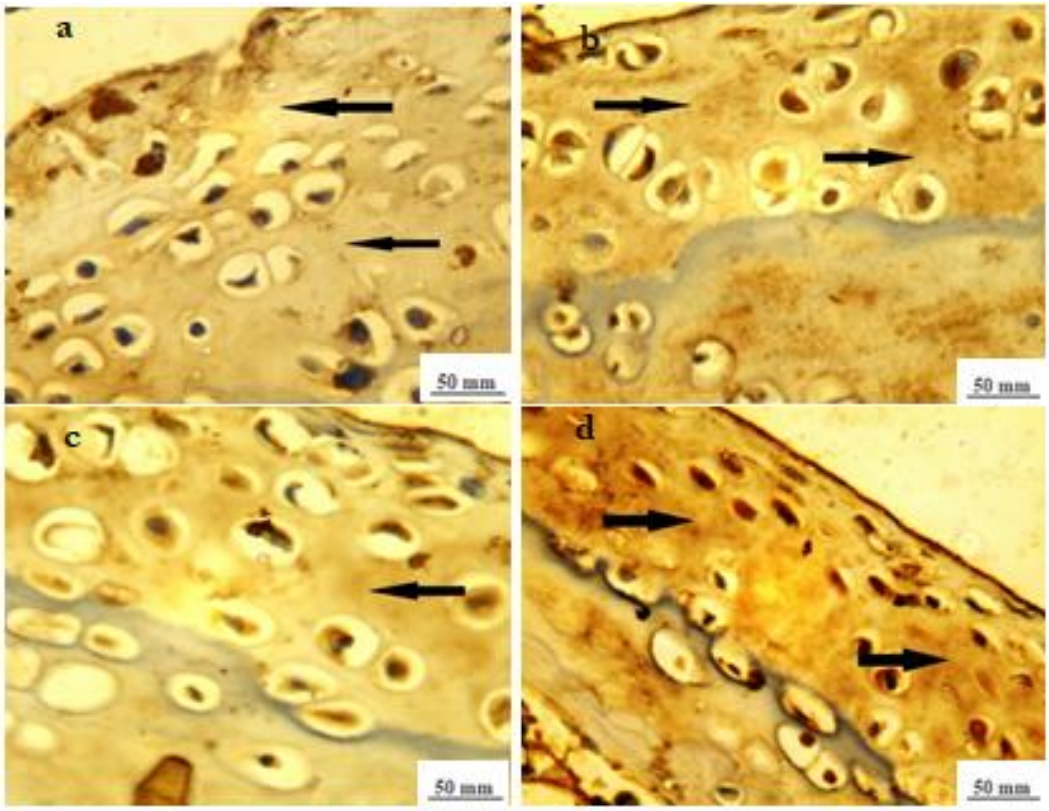


**Fig. 4. A)** Immunohistochemistry of aggrecan in the articular cartilage of the mild osteoarthritis group showing a reduction of aggrecan immunostaining intensity in the entire articular cartilage (arrow). X1000. **B)** Immunohistochemistry of aggrecan in the articular cartilage of the severe osteoarthritis group showing a marked decrease in the immunostaining (arrow). X1000. **C)** Immunohistochemistry of aggrecan in the articular cartilage of the mild osteoarthritis treated group showing reduction in aggrecan immunostaining intensity was less pronounced (arrow). X1000. **D)** Immunohistochemistry of aggrecan in the articular cartilage of the severe osteoarthritis treated group showing an increase in aggrecan immunostaining (arrow). X1000.

**Histomorphometric and statistical results:**

***Articular cartilage thickness:***

The mean articular cartilage thickness in the mild and severe osteoarthritis groups was significantly lower than in the control group. The severely treated group showed a significant decrease compared to the control. When compared with the mild osteoarthritis group, the mild treated group showed a non-significant decrease. By comparing of the severe osteoarthritis group, the severe treated group showed a non-significant increase of the articular cartilage thickness (Histogram is shown in Fig. 5).

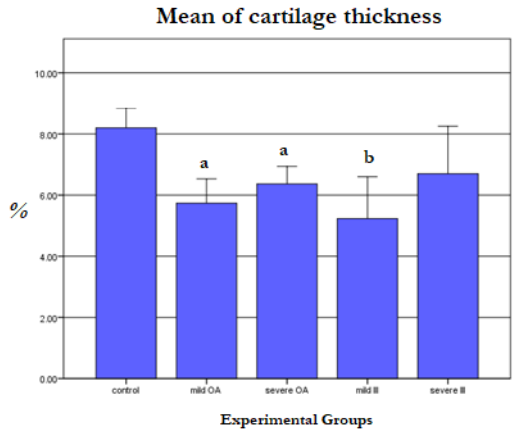


**Fig. 5. Histogram represents the mean cartilage thickness (µm) in all experimental groups.** ([a]); significant changes when compared with the control group when $p$ is $\leq 0.05$. ([b]); significant changes when compared mild osteoarthritis group when $p$ is $\leq 0.05$

***The number of chondrocytes:***

A highly significant decrease in the number of chondrocytes in the mild treated group compared with the control group and a non-significant rise in the mild osteoarthritis group. In the severe treated group, there was a non-significant increase compared to the control and a highly significant increase compared to the severe osteoarthritis group (The histogram is shown Fig. 6).

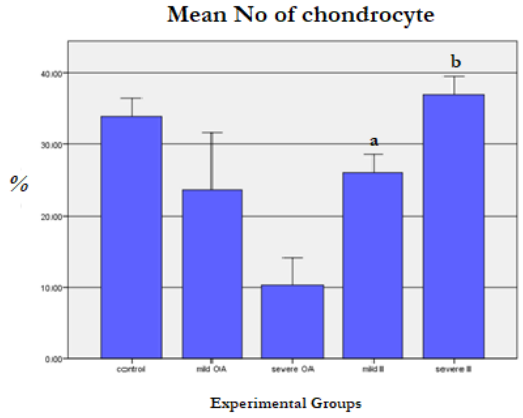


**Fig. 6. Histogram represents the mean number of chondrocytes in all experimental groups.** ([a]); significant changes when compared with the control group when $p$ is $\leq$ 0.05. ([b]); significant changes when compared with severe osteoarthritis group when $p$ is $\leq$ 0.05

***The mean area percentage of aggrecan immunostaining:***

In both the mild and severe untreated osteoarthritic groups, the mean area percentage of aggrecan was significantly lower than in the control group. When compared to the control, the mean area percentage of aggrecan in both treated groups were significantly lower. A non-significant rise in the mild treatment group compared to the mild osteoarthritis group. When compared to the severe osteoarthritis group, there was a highly significant increase in the severe treated group (Histogram is shown in Fig. 7).

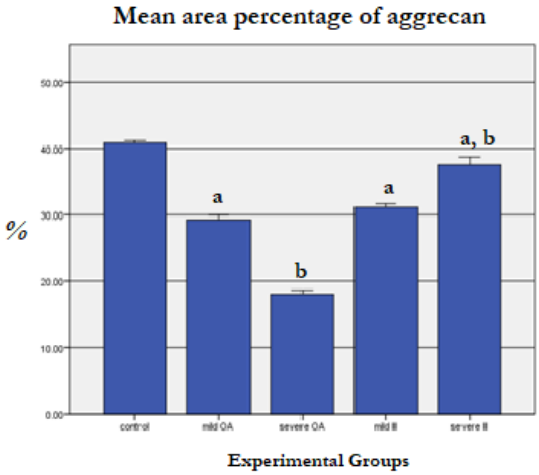


**Fig. 7. Histogram represents the mean area percentage of aggrecan immunostaining in all experimental groups.** ([a]); significant changes when compared with the control group when $p$ is $\leq$ 0.05. ([b]); significant changes when compared with severe osteoarthritis group when $p$ is $\leq$ 0.05

**Table 1. Mankin's total score is the sum of the scores for cartilage structure, cellular abnormalities, tideline, and toluidine blue matrix staining.** A total score of 14 indicates extensive cartilage destruction, whereas a score of 0 indicates normal cartilage.

| Cartilage structure | Score |
|---|---|
| Normal | 0 |
| Surface irregularities | 1 |
| Pannus and surface irregularities | 2 |
| Clefts to a transitional zone | 3 |
| Clefts to radial zone | 4 |
| Clefts to calcified zone | 5 |
| Complete disorganization | 6 |
| **Cellularity** | **Score** |
| Normal | **0** |
| Diffuse hypercellularity | 1 |
| Cloning | 2 |
| Hypocellularity | 3 |
| **Toluidine blue staining** | **Score** |
| Normal | 0 |
| Slight reduction | 1 |
| Moderate reduction | 2 |
| Severe reduction | 3 |
| Absent | 4 |
| **Tidemark integrity** | **Score** |
| Intact | 0 |
| Crossed by blood vessels | 1 |

**Table 2. Total histopathological lesions score (Mankin score) of knee cartilage from control, mild untreated OA, severe untreated OA, mild treated OA, and severely treated OA animal groups**. Data expressed as mean±SD. $P_1$ vs. control group; $P_2$ vs. Mild OA group; $P_3$ vs. severe OA group, $P_4$ vs. mild treated OA group, and NS; non-significant. Statistical analyses were performed by one-way analysis of variance with Tukey's post hoc test (P <0.001).

| | Mean±SD | P1 | P2 | P3 | P4 |
|---|---|---|---|---|---|
| **Control** | 0.00 | | | | |
| **Mild OA** | 7.4±1.02 | <0.001 | | | |
| **Severe OA** | 11.7±1.3 | <0.001 | <0.001 | | |
| **Mild treated OA** | 4.2±1.3 | <0.001 | <0.001 | NS | |
| **Severe treated OA** | 6.5±0.6 | <0.01 | <0.01 | <0.001 | <0.001 |

## Discussion

OA is a degenerative and inflammatory joint condition that causes joint dysfunction and loss. It causes significant damage, articular cartilage degeneration, bone enlargement at the margins (i.e. osteophytes), and subchondral sclerosis in joints (McCarty and Koopman, 1993; Shariatzadeh et al., 2019).

The amount of MIA injection was found to be a key determinant in the course of OA. The degree of histopathological alterations was observed to be dose-dependent in prior investigations (Bove et al., 2003; Takahashi et al., 2018; Udo et al., 2016).

Bove et al., (2003) and Guingamp et al., (1997) showed that 0.2 mg of MIA as the representative low dose in the MIA model and 1.0 mg of MIA as the representative high dose in the MIA model. Furthermore Lampropoulou-Adamidou et al., (2014) reported that in rats, 1.0 mg of MIA was the maximal effective dose for inducing OA.

Many researchers have observed histological alterations in the articular cartilage of the tibiofemoral joint following MIA injection, including articular cartilage degradation and subchondral bone necrosis, as well as chondrocyte necrosis (Cifuentes et al., 2010; Moon et al., 2012; Saito et al., 2017). The same histopathological changes were detected in the study after OA induction in the knee joint.

By stimulating cartilage immune responses, copper aids in the regeneration of both sub-chondral bone and articular cartilage, resulting in the restoration of the osteochondral interface and the repair of cartilage lesions (Djoko et al., 2015). Copper also aids in the regeneration of both sub-chondral bone and articular cartilage by boosting cartilage immune responses, resulting in the restoration of the osteochondral interface and the repair of cartilage lesions (Lin et al., 2019).

The current study's findings demonstrated that both mild and sever treated groups showed lesser degenerative changes, which appeared nearly normal except for few shrunken chondrocytes, moreover it showed increased and marked cellularity with decreased degenerated chondrocytes with faint tidemarks. Copper minimizes cartilage injury and promotes the development and proliferation of chondrocytes by suppressing the inflammatory response (Yassin et al., 2015; Chen et al., 2020). Furthermore, copper supplementation in meals has been shown to reduce the severity of osteoarthritis and other developing cartilage lesions, owing to improved collagen cross-linking and increased collagen type II production (Yuan et al., 2011).

Also, the obtained results agreed with previous reviews as it shows reduction in aggrecan immunostaining intensity was less pronounced in both mild and severe osteoarthritis treated group. Moreover, the articular cartilage thickness in both mild and sever treated group showed a non-significant decrease. Interestingly, both the moderate and severe treated osteoarthritis groups showed a non-significant increase in the mean number of chondrocytes in the articular cartilage. Copper's cartilage-protecting mechanism may be due to Cu2anti-catabolism+'s which prevents the proteoglycan degradation of cartilage matrix due to the release of oxidants (Gee et al., 2007).

Inflammatory disorders are known to cause serum copper levels and ceruloplasmin activity to be much higher than usual. Copper is also one of the endogenous modulators of the inflammatory response (Milanino et al., 1985).

Lewis et al., (1982) and Rainsford, (1982) reported that the administration of copper complexes has the ability reduce the symptoms of chronic inflammation, especially adjuvant arthritis, in laboratory animals.

The Mankin grading system was used to determine changes in the structure of the proteoglycan aggrecan (PG) of articular cartilage. The findings revealed a significant increase in the mean area percentage of aggrecan immunostaining, as well as Mankin score revealed highly significant less degenerative changes in the articular cartilage, which was associated with better articular cartilage preservation in both treated groups (Lark et al., 1997).

## Conclusion

Overall, the findings indicated that in pathological changes involving aggrecan or histopathological abnormalities in the articular cartilages in OA of the knee can be classified as mild or severe. Furthermore, a lack of copper in the diet causes a change in copper levels, which can lead to joint injury, including bone and cartilage destruction. Copper consumption that is well balanced has a positive impact on OA.

## Author contributions

AZS., MSA., AYN and OS conceived and designed the study. OS., AAM and MY conducted the experiment and collected the data. NAMM and RIE performed histopathological and immunohistochemistry studies. OS., AA., NAMM., HM and RIE organized and analyzed the data. AAS and AAM interpreted the data, wrote the paper, and revised the final draft. All authors have read and agreed to the published version of the manuscript.

**Conflict of interest statement**

The authors declare that they have no conflict of interest.

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