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Investigation of the Ameliorating Effect of Copper Albumin Complex on Lysyl oxidase in monosodium iodoacetate -Induced Knee Osteoarthritis in Rats

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Abstract

Knee osteoarthritis (KOA) is a common type of joint degeneration which causes progressive damage of the joint structure and has less therapeutic options. It has been found that oral consumption of Copper Albumin Complex as anti-inflammatory drug has a positive effect on the treatment of joint deterioration. The present study aimed to investigate the effect of oral administration of Copper Albumin Complex (cu-albumin complex) on Lysyl oxidase (LOX) which acts as a protective factor in KOA. Fifty adult albino rats were divided into 3 groups: negative control (10 normal rats); positive control (20 rats with KOA which left without induction treatment); and treated group (20 rats with KOA which treated with administration of copper albumin complex). Treated and untreated arthritic groups were subdivided equally into mild and severe groups (10 rats for each) according to the severity of clinical signs. KOA was induced by intra-articular injection of monosodium iodoacetate (MIA). At the experimental end, the joints were examined histopathologically and immunohistochemically after cervical dislocation of rats. It was observed that the treatment with CU- was effective in reducing disease severity and in improvement of Lysyl oxidase KOA. It was concluded that Copper albumin complex has a positive effect in the improvement of LOX of Knee joint cartilages of rats affected by osteoarthritis (OA).

Keywords: Knee; Osteoarthritis; Lysyl oxidase; Copper-albumen complex; Cartilage.

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Introduction

Osteoarthritis (OA) is the most common degenerative joint disease, leads to structural damage and ultimately loss of function and disability. Progressive, irreversible destruction of the joints in OA is driven by defective cartilage extracellular matrix (ECM) remodeling and the loss of chondrocytes due to apoptosis (Zamli and Sharif, 2011). Knee pain and symptomatic knee OA have increased in prevalence (Nguyen et al., 2011), and similar manifestations of the disease process are seen in temporomandibular joint (TMJ) disorders (Scrivani, Keith and Kaban, 2008). The risk factors of osteoarthritis include older age, obesity, previous joint injury, joint deformity, and inherited factors (Van den Berg, 2011; Razek and El-Basyouni, 2016). While osteoarthritis (OA) affects the older population leading to disability with progressive age (65+), there remain few therapeutic options (Bais and Goldring, 2017).

Lysyl oxidase (LOX) is a copper-dependent quinone-cofactor amine oxidase secreted by various cell types and which has function in ECM remodeling and collagen cross-linking (Harris, 1976; Alshenibr et al., 2017; Tang et al., 2017). LOX activity is required for the formation of immature and mature pyrodinoline (PYR) cross-links in native and engineered cartilage. Hypoxia-induced LOX increases PYR crosslinks and tensile properties of the articular cartilage, knee meniscus, patellar tendon, and anterior and posterior cruciate ligaments, while exogenously applied LOX proteins are capable of enhancing collagen cross-linking and cartilage tissue functional properties (Makris, Responte, et al., 2014).

Previous studies have demonstrated the vital roles of LOX in normal chondrocyte function (Sanada et al., 1978; Ahsan et al., 1999), which may be correlated with the pathogenesis of aging-associated osteoarthritis (Pokharna et al., 1995) (Pokharna et al., 1995).

Moreover, LOX has recently been demonstrated as a potential chondro-protective factor in aging related joint osteoarthritis, mainly through inducing anabolic gene expression and attenuating catabolic genes (Bais and Goldring, 2017; Tashkandi et al., 2019). In osteoarthritis, LOX was found to exert potential therapeutic effects in cartilage degeneration by enhancing extracellular matrix ECM synthesis (Makris, MacBarb, et al., 2014).

The present study aimed to evaluate the potential of copper albumin complex consumption on LOX during KOA rats' model.

Materials and methods

Ethical approval

The study protocol was approved by the Animal Research Committee of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt (Approval no. 32/2022).

Animals

Fifty male albino rats (12-week-old; mean mass, 140 + 10 g) were used in the study. The Rats were housed for 10 days before the experiment to acclimate them to the environment in a room with standard environmental condition (24+ 2℃ temperature, and 50+ 5 % humidity) with a 12 h light/ dark cycle. The rats fed on a ratio of 14 % protein and water was provided ad-Libidum. After the adaptive

period, the osteoarthritis was induced in rats by single intra-articular injection of monosodium iodoacetate (Guzman et al. 2003; Udo et al. 2016; Takahashi et al. 2017; Xu et al. 2020) as 3 mg of MIA (Sigma Aldrich; St. Louis, MO, USA) was dissolved in 50 µl sterile saline for each Kg/ B.W and injected in the right knee anesthetized rats. The hair on the right knee joints was removed and the area was disinfected with 70% alcohol. An incision was created at the center of knee to expose the patellar ligament while the rats were positioned on their backs and the right leg was 90˚ flexed as the injection was performed below the patella after locating the patellar ligament (Takahashi et al., 2018).

Experimental groups

After a week of experimental osteoarthritis induction, rats were randomly grouped into five groups as following;

Negative control: included on 10 normal rats without induction of OA.

Positive control: included on 20 osteoarthritic rats which left without treatment and were divided equally into mild and severe not treated subgroups according to the detected clinical signs which are pain, swelling and, lameness.

Treated group: included on 20 osteoarthritic rats which were divided equally into mild and severe treated groups according to severity of clinical signs and treated orally with copper albumin complex (obtained from 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) for a month at a dose of 1 ml/kg B.W daily after suspension of 817µg/kg copper albumin complex in water (Taha et al., 2022).

Joints examination

The swelling of the right knee joint was measured once weekly for a month during the experimental period by using a digital caliper. Also the joint mobility, motor function and pain were assessed once a week by the accelerating rotarod apparatus (Ugo Basile, Varese, Italy, Model 7750) according to Vonsy et al. (2009).

Histopathologic examination of the right knee joint

The animals were euthanized by cervical dislocation after the end of the experiment and the right knee from all groups were excised, washed with saline solution and decalcified by 10% ethylene diamin tetraacetic acid (EDTA) solution for a week. After the decalcification, joints were fixed in 4% neutral buffered formalin for 48 hrs. Followed routine histopathological preparation by cutting of samples into 5µm thick using Microtome and stained with Masson trichrome stain (Schmitz et al., 2010; Suvarna, Layton and Bancroft, 2018). The prepared slides were examined under the microscope. All slides were scored according to guidelines of the 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-lysyl oxidase primary antibody (Abcam, England) with concentration of 1:50 was added at 4°C for 24 hrs. The secondary

antibody was conjugated with horse radish peroxidase (Abcam) for 60 min, followed by diaminobenzidine (DAB) (DakoCytomation, Denmark), and stained by hematoxyline. The samples were examined using a light microscope and the area of immunostaining was measured by Image J.

Statistical analysis

The results were statistically analyzed using SPSS (Statistics package version 17.0 SPSS Chicago, IL, USA) and expressed as means ± standard deviation and one-way analysis of variance was used for quantitative differences between the values. Turkey test was used for multiple intergroup comparisons. A P-value of <0.05 was considered to be statistically significant.

Results

Masson trichrome staining

Masson trichrome staining was used for evaluation of the collagen of the cartilage matrix. Masson trichrome commonly stains the cartilage matrix green, the nuclei dark blue, and the zone of calcifying cartilage red. In the control group, the articular cartilage matrix well stained with Masson trichrome (green color), reflecting the normal content of collagen fibers (Fig.1A). On the other hand, the articular cartilage of the mild osteoarthritic group showed a slight reduction of Masson trichrome–stained area for collagen with the appearance of slight red color reflecting a mild reduction of collagen fibers in the matrix (Fig.1B). In the severe osteoarthritic group, a marked reduction of collagen was observed (Fig.1C). Interestingly, the matrix of the mild osteoarthritic treated group revealed an increase in the Masson trichrome–stained area for collagen with a reduction in the red color (Fig.1D). In the severe osteoarthritic treated group, the articular cartilage's collagen content increased (Fig.1E).

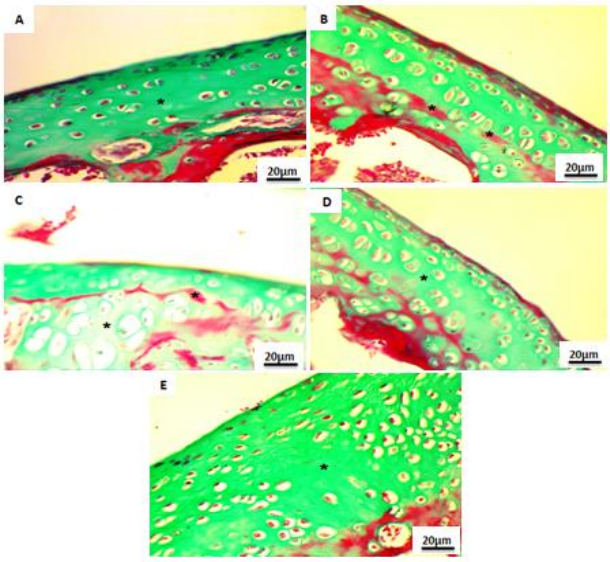


Figure 1: Photomicrograph of Masson trichrome–stained sections of the knee joint's articular cartilage: **A)** The control group showed the well-stained articular cartilage's collagen with Masson trichrome (green color) (*) which reflected the regular content of collagen fibers. **B)** The mild osteoarthritis group showed a slight reduction of Masson trichrome–stained area for collagen with the appearance of a slight red color (*). **C)** The severe osteoarthritis group showed a marked reduction of Masson trichrome–stained area for collagen (*). **D)** The mild osteoarthritis treated group revealed an increase in the Masson trichrome–stained area for collagen, with a reduction in the red color (*). E) The severe osteoarthritis treated group, showed an increase in the articular cartilage's collagen content (*). X 200.

Immunohistochemical staining for collagen type II:

Immunohistochemical staining for expression of collagen type II fibers in the articular cartilage of the control group revealed strong immunostaining intensity (brown color) reflecting the dense and uniform distribution of collagen type II fibers in the matrix (Fig. 2A). On the other hand, immunohistochemical staining of collagen type II fibers of articular cartilage

of mild osteoarthritis group revealed a decrease in the immunostaining intensity reflecting a decrease of collagen type II fibers in the matrix of the articular cartilage (Fig. 2B). In the severe osteoarthritis group, a marked decrease in the immunostaining was observed in the articular cartilage (Fig.2C). However, collagen type II fibers expression was stronger in the treated group than in the osteoarthritis group. In the mild osteoarthritis treated group (Fig.2D) and the severe treated group (Fig.2E) showed increase in the immunostaining intensity for collagen type II.

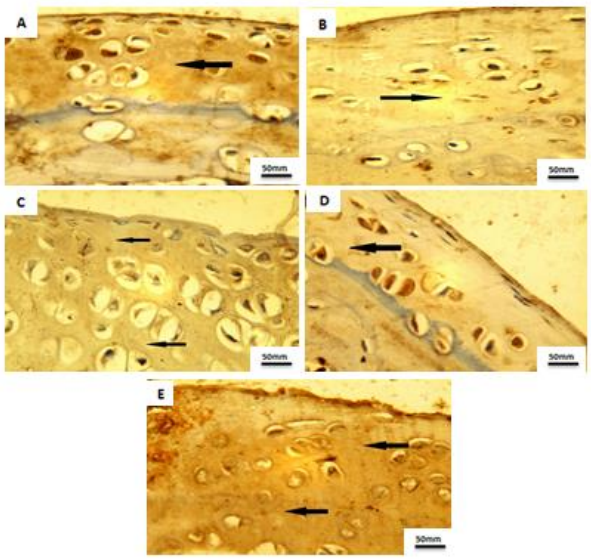


Figure 2: Immunohistochemistry of stained sections for collagen type II of the knee joint articular cartilage: **A)** The control group showed strong immunostaining intensity for collagen type II in articular cartilage (arrow). **B)** The mild osteoarthritis group showed a decrease in the immunostaining intensity for collagen type II in articular cartilage (arrow). **C)** The severe osteoarthritis group showed a marked decrease in the immunostaining of collagen type II in the matrix (arrow). **D)** The mild treated osteoarthritis showed moderate immunostaining intensity for collagen type II in articular cartilage (arrow). **E)** The severely treated osteoarthritis showing moderate immunostaining of collagen type II in articular cartilage (arrow). X1000.

Immunohistochemistry of lysyl oxidase

In the control group, there was a positive reaction in the cytoplasm of chondrocytes (Fig.3A). In the mild untreated osteoarthritis group, a decrease in the positive reaction was observed (Fig.3B). In the severe untreated osteoarthritis group, there was a marked decrease in the positive reaction in the chondrocytes (Fig.3C). In the mild treated group, an increase in the positive reaction was observed (F.3D). There was a marked increase in the positive reaction in the severe treated group (Fig.3E).

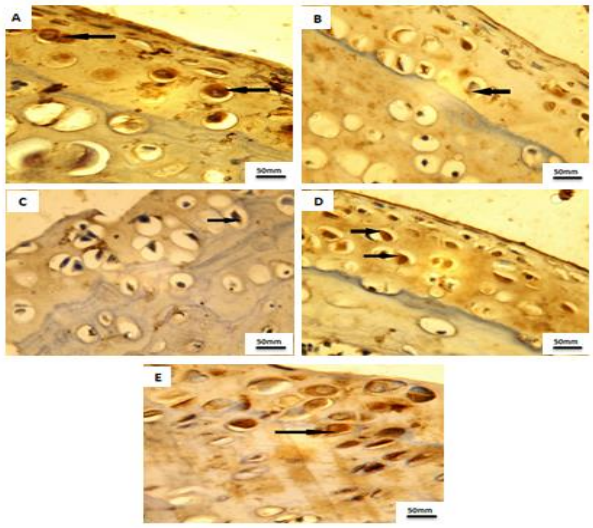
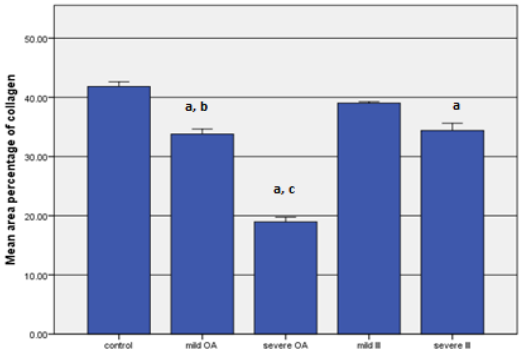


Figure 3: Immunohistochemistry of stained sections for lysyl oxidase of the knee joint articular cartilage: **A)** The control group showed a positive reaction in the cytoplasm of chondrocytes (arrow). **B)** The mild untreated osteoarthritis group showed a decrease in the positive reaction (arrow). **C)** The articular cartilage of the knee joint of the severe untreated osteoarthritic group showed a marked decrease in the positive reaction in the chondrocytes (arrow). **D)** The mild treated group showed an increase in the positive reaction (arrow). **E)** The severe treated group showed a marked increase in the positive reaction (arrow). X1000

Histomorphometric and statistical results

The mean area percentage of collagen fibers stained by Masson trichrome

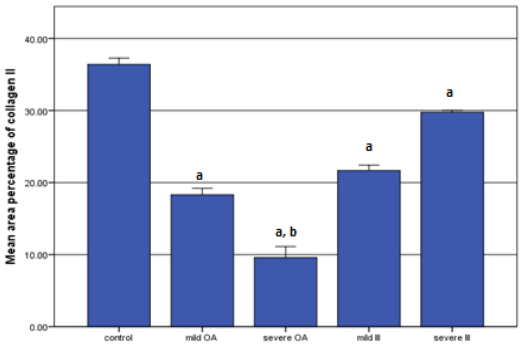
The mean area percentage of collagen fibers in the mild and severe untreated osteoarthritis group showed a highly significant decrease compared to the control. The mean area percentage of collagen fibers in the mild treated group showed a non-significant decrease compared to the control and a highly significant increase compared to the mild osteoarthritis group. In the severe osteoarthritis treated group, a highly significant decrease compared to the control group and a highly significant increase compared to the severe osteoarthritis group (Histogram 1).



Histogram 1: represents the mean area percentage of collagen fibers in all experimental groups. ([a]); significant changes when compared with control group when *p* was ≤ 0.05. ([b]); significant changes when compared with mild treated osteoarthritis group when *p* was ≤ 0.05. ([c]); significant changes when compared with severe treated osteoarthritis group when *p* was ≤ 0.05

The mean area percentage of collagen II immunostaining

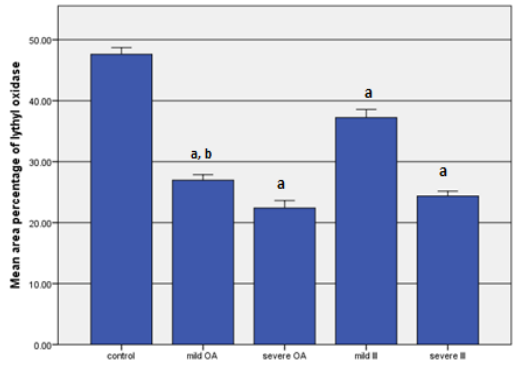
The mean area percentage of collagen II in the mild and severe untreated osteoarthritis group showed a highly significant decrease compared to the control. The mean area percentage of collagen II in both treated groups showed a highly significant decrease compared to the control. In the mild treated group, there was a non-significant increase compared to the mild osteoarthritis group, while in the severe treated group, a highly significant increase compared to the severe osteoarthritis group was observed (Histogram 2).



Histogram 2: represents the mean area percentage of collagen II immunostaining in all experimental groups. (a); significant changes when compared with control group when p was ≤ 0.05. (b); significant changes when compared with severe treated osteoarthritis group when p was ≤ 0.05.

The mean area percentage of lysyl oxidase immunostaining:

The mean area percentage of lysyl oxidase in the mild and severe untreated osteoarthritis groups showed a highly significant decrease compared to the control. The mean area percentage of lysyl oxidase in both treated groups showed a highly significant decrease compared to the control. In the mild treated group, there was a highly significant increase compared to the mild osteoarthritis group. In contrast, in the severe treated osteoarthritis group, there was a non-significant increase compared to the severe osteoarthritis group (Histogram 3).



Histogram 3: represents the mean area percentage of lysyl oxidase immunostaining in all experimental groups. (a); significant changes when compared with control group when p was ≤ 0.05. (b); significant changes when compared with mild treated osteoarthritis group when p was ≤ 0.05.

Histological scoring (Mankin score) (Table 1):

The Mankin score of the control group was 0. The examination of the stained sections of the osteoarthritis group showed a highly significant articular cartilage damage (irregular notched surface, hypocellularity, severe reduction in the matrix staining intensity, and invisible tidemark) as compared to the control group, with a score of 7.40± 1.02 in the mild osteoarthritis group and 11.7±1.3 in the severe osteoarthritis group. The examination of the stained sections of the treated osteoarthritis group revealed highly significant less degenerative changes in the articular cartilage as compared to the osteoarthritis group, with a score of 4.20±1.3 in the mild treated group and 6.5±0.6 in the severe treated group, indicated that the treated groups was associated with better preservation of articular cartilage (Table 2).

Table 1: Manikin's total score (1-14) is the sum of the scores for cartilage structure, cellular abnormalities, tideline, and toluidine blue 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 hyper-cellularity	**1**
Cloning	**2**
Hypo-cellularity	**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: shows the total histological lesions score (Mankin score) of knee cartilage in the control, moderate 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).

Group	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	6.5±0.6	<0.01	<0.01	<0.001	<0.001

Discussion:

OA is a degenerative joint condition that is primarily caused by aging. Inflammation plays a crucial role in the etiology of OA since affected patients are mostly identified by cartilage dysfunction. It is debatable whether inflammatory mediators are primary or secondary regulators of cartilage degradation and disrupted repair processes in OA, even though there are numerous well-described molecular pathways and mediators implicated in inflammation (Tramś et al., 2022).

The present study was carried out on osteoarthritis of knees joints in rats model as they are the most commonly affected joints with OA (Burn et al., 2019; Sit et al., 2019), where more than 10% of the population is thought to be affected by knee osteoarthritis, with a lifetime risk of 45%. Current recommendations encourage maintaining a healthy weight, therapeutic exercise, pharmacological treatment (oral non-steroidal anti-inflammatory drugs, paracetamol, and opioids), and the use of mechanical aids (walking aids, braces, orthoses). However, the benefits of these treatments are frequently just temporary. Intra-articular corticosteroids are often advised, but only for temporary pain relief due to their short-lived advantages (Rodríguez-Merchán, 2022). Pain, stiff joints, functional impairment, and even incapacity are all symptoms of KOA, which places a significant strain on healthcare services (Abbasi, 2017).

The study aimed to investigate the effect of oral supplementation of copper-albumen complex on LOX in MIA-induced knee osteoarthritis in rats. Fonsi et al. (2020) stated that, rat model of MIA-induced KOA is appealing as animal can quickly develop the illness condition, which appeared to accurately resemble OA in people. Also, Gowler et al. (2020) mentioned that surgical models of OA in rodents frequently have a quick onset When it comes to the mechanistic value and translational validity. Rat pain and motor activity, and knee histopathology all these criteria were used to evaluate the development of OA symptoms in rats (Fonsi et al., 2020). Smith-Mungo and Kagan, (1998) recorded that LOX is a copper-dependent amine oxidase which plays a crucial role in the biogenesis of connective tissue matrices by crosslinking the extracellular matrix proteins, collagen and elastin. Also, Alshenibr et al. (2017) mentioned that LOX up-regulated in cartilage affected by OA, this may be a protective response that promotes anabolism while inhibiting specific catabolic responses in the pathophysiology of OA. Further, LOXL2 also was found to

have a novel anabolic function in OA cartilage (Alshenibr et al., 2017). Kagan and Trackman (1991) mentioned that, the covalent cross-linkages that stabilize the elastin and collagen fibers are produced when peptidyl lysine is oxidized to α-aminoadipic-δ-semialdehyde by the enzyme lysyl oxidase. Alterations in the integrity of the extracellular matrix play an important role in osteoarthritis. Two major mechanisms of collagen cross-linking may contribute to collagen aging in cartilage. The first is based on lysyl oxidase-mediated collagen crosslinking. Lysyl oxidase catalyzes the oxidative deamination of amino groups of lysyl and hydroxylysyl residues. The aldehyde formed condenses with the amino group of other lysyl/hydroxylysyl residues to form Schiff bases (Pokharna et al., 1995).

Copper is a vital trace mineral which required sustaining our body's physiological balance and antibiosis (Skaar and Raffatellu, 2015; Bost et al., 2016). It was reported that, copper is essential for both cellular and humoral immunity (Djoko et al., 2015). Additionally, acute phase responses to diseases including infection, inflammation, and other illnesses all clearly result in an increased metabolism of copper (Tapiero, Townsend and Tew, 2003). Copper stimulates the production of inflammatory cytokines and hypoxia-inducible factor, which in turn enhances the synthesis and secretion of ceruloplasmin (Linder, 2016). Copper plays a key role in the production of numerous cellular enzymes, including the copper-containing superoxide dismutase, cytochrome oxidase, and lysyl oxidase, in healthy tissues, especially cartilage (Fraga, 2005). Particularly, it has been demonstrated that lysyl oxidase, a copper-dependent amine oxidase, is a crucial enzyme for collagen cross-linking and additionally facilitates the synthesis of cartilage (Huang et al., 2016; Alshenibr et al., 2017). Additionally, copper imbalance could also weaken bones and promote osteoarthritis (Alshenibr et al., 2017). Copper has also been shown to have beneficial effects on osteogenesis and anti-arthritis (Goggs et al., 2005; Wang et al., 2014).

When comparing the results obtained between the experimental groups of animals, a sharp difference in microscopic changes with control group was determined, that indicated the presence of degenerative-dystrophic changes in the knee joints of mild and severe osteoarthritis group. The most pronounced intensity of pathological changes was determined severe osteoarthritic group which confirms the adequacy of the performed OA model (Dmitriy Nosivets, Eulalia Montell and Valentine Opryshko, 2021).

The treated osteoarthritis groups showed histopathological improvement after oral treatment with copper- albumin complex, which indicated that the positive therapeutic effect of copper in treatment of osteoarthritis weather mild or sever. This result was supported by an experimental study carried out by Yassin et al. (2015) on the use of copper indomethacin (Cu-Indo) gel preparation on monosodium iodoacetate (MIA) induced arthritis of the knee joint of rats proved that copper possess anti-inflammatory activity against osteoarthritis.

The Mankin technique described the major histopathological scores used to identify the alterations in OA as the integrity of tidemarks, cellularity, cartilage

structure, and proteoglycan staining (Little et al., 2010).

The present study revealed that highly significant articular cartilage damaged in osteoarthritis groups compared with normal group. While, the stained sections of the treated osteoarthritis group showed significantly decreased degenerative changes in comparing with the osteoarthritis group in the articular cartilage (Lark et al., 1997), these indicated that the copper-albumin complex assisted in decreasing the degenerative effect of the articular cartilages.

Conclusion

Copper albumin complex has potent anti-inflammatory activity against knee osteoarthritis in the MIA-treated rat model, as it improved the joint cartilage health and decreased the deteriorative effect of osteoarthritis in the cartilage structure when orally supplemented.

Author contributions

OMG., 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., MAK., SSF., and MMS organized and analyzed the data. 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.

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