

THE IMPACT OF ANTIOXIDANTS ON INFLAMMATION AND OXIDATIVE STRESS MARKERS IN OSTEOARTHRITIS RAT MODEL: SCANNING ELECTRON MICROSCOPE INSIGHTS

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ABSTRACT

Osteoarthritis (OA) is characterized by degradation of matrix and destruction of articular cartilage. Articular chondrocytes are solely responsible for the production and maintenance of the extracellular matrix. Therefore, chondrocyte disruption is implicated in cartilage degeneration. Numerous studies have shown that antioxidant treatments are promising therapeutics in cases of OA. This study was designed to examine whether vitamin E protects rat articular chondrocytes against increased inflammatory markers and oxidative stress and prevents cartilage destruction in mono-iodoacetate-induced osteoarthritis rat model. Data showed that osteoarthritis group showed a significant increase in inflammatory markers, Tumor Necrosis Factor- α (TNF- α) (38 ± 1 ng/mL), Interleukin-6 (IL-6) (253 ± 15 ng/mL) and oxidative stress marker, Super Oxide Dismutase (SOD) (14 ± 1 ng/mL) compared to control (18 ± 1 ng/mL), (121 ± 23 ng/mL) and (8 ± 1 ng/mL) respectively. Opposite trend was found when animals were treated with vitamin E where TNF- α (27 ± 2 ng/mL) and SOD (10 ± 1 ng/mL) declined significantly. Electro-microscopic examination documented the above results and showed improvement of knee joint after administration of vitamin E. This study supported the notion that OA is a multi factorial complication, caused by inflammation and increased oxidative stress. Administration of vitamin E decreased the markers of inflammation and oxidative stress as well as improved ultra-structure of the knee joint in acute OA animal model. However, further work is needed to validate reliability in human patients suffering from osteoarthritis.

Keywords: Antioxidants, Vitamin E, Electron Microscope, Osteoarthritis

1. INTRODUCTION

Osteoarthritis is the most common joint disease and is among the most frequent health problems for middle aged and older people. It is characterized by articular cartilage destruction and osteophyte formation (Namazi and Majid, 2005).

Since articular cartilage is a tissue type that is poorly supplied by blood vessels, nerves and lymphatic system, it has a very limited capacity for repair after injury.

The experimental models of OA are of both scientific and applied importance. Sometimes, this is the only means to confirm a working hypothesis. The reproduction of all disorders and symptoms of OA is

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however challenging, so a model possessing the most important signs of natural disease and that could be reproduced, is good enough for the purpose of research (Williamson and Marshall, 2014). The interest towards reproduction of OA model of the knee is due to its anatomical features and the increasing prevalence of degenerative events (Williamson and Marshall, 2014). The Metabolic inhibitor sodium Mono-Iodoacetate (MIA) destructs the joint cartilage by blocking glyceraldehyde-3-phosphate dehydrogenase in chondrocytes and inhibits glycolysis, which leads to rapid depletion of ATP, cell death and decreases synthesis of proteoglycans for the articular matrix. MIA-induced degenerative processes, similar to OA lesions in humans which appears within 6 to 8 weeks (Combe *et al.*, 2004; Bove *et al.*, 2006).

The main clinical sign of OA is joint pain, which is associated with the release of inflammatory mediators (Bove *et al.*, 2006). One of the sources of the pain is considered to be the local inflammation in knee joints, such as that in the synovial membrane (Pearle *et al.*, 2007) followed by the release of several cytokines such as TNF- α , Interleukins (ILs) such as IL-1 and IL-6 and nerve growth factor. These mediators further contribute to OA pathogenesis by increasing cartilage degradation and inducing hyperalgesia (Collazos *et al.*, 2007).

TNF- α activates sensory neurons directly via its receptors and initiates a cascade of inflammatory reactions through the production of ILs such as IL-1 and IL-6 (Hargadon *et al.*, 2012). At low concentrations in tissues, TNF- α has been called a guard cytokine as it initiates the defense response to local injury and augment of host defense mechanisms against infection (Feldmann and Steinman, 2005). At high concentrations, TNF- α leads to inflammation and organ injury. Many different immune and non-immune cell types can produce TNF- α , including macrophages, T cells, mast cells, granulocytes, natural killer cells, fibroblasts, neurons and smooth muscle cells (Smookler *et al.*, 2006).

TNF is generally considered to be a pro-inflammatory cytokine, along with ILs and other cytokine (Tracey *et al.*, 2008). In particular, IL-6 is reported to have a complex role in OA pathogenesis by initiating inflammatory responses such as the production of tissue inhibitors of metalloproteinases-1, which may act to limit cartilage damage through negative feedback (Anggraeni, 2011). Receptor-mediated effects of TNF can lead alternatively to activation of nuclear factor kappa-B (NF- κ B) depending on the metabolic state of the cell (Eissner *et al.*, 2004). The primary pathway leads to activation of NF- κ B, leads to caspase-8-and caspase-

3-dependent apoptosis and the release of cytochrome C from mitochondria (Schneider-Brachert *et al.*, 2004). Apoptosis leads to leukocyte migration and attack on parenchymal cells (Eipel *et al.*, 2004) thereby establishing a vicious circle with aggravation of leukocytes inflammation and cell death (Le Minh *et al.*, 2007).

The articular cartilage is considered a hypoxic tissue, once it is an avascular tissue. However, the implications of this hypoxic environment are poorly understood at the molecular level. A topic of great interest is the role of changes in oxygen levels during cartilage degeneration process. Elevated production of Reactive Oxygen Species (ROS) and/or depletion of antioxidants have been observed in a variety of pathological conditions, including inflammatory joint diseases. ROS catalyze lipid peroxidation and tissue injury (Zangiabadi *et al.*, 2011). It is possible though that reactive species may be involved in the pathophysiological process of OA. Nevertheless, the systemic implications on OA of ROS production are still unknown and require investigation (Henrotin *et al.*, 2005).

Although several therapies have been used for OA, no widely accepted treatments have been established, with the exception of arthroplasty. Currently, the most effective treatment for OA, besides arthroplasty, is autologous chondrocyte transplantation. However, this treatment has several limitations, including the need to use neighboring healthy donor cartilage, difficulty in treating large-scale defects, limited expansion capacity of primary chondrocytes and the need for a periosteal patch to maintain engineered cartilage. In addition, in most cases only 30-40% of the defect regenerates articular cartilage, with the remaining defect being filled with fibro cartilage (Fickert *et al.*, 2014). In light of these limitations, it is important to find other lines of treatment that can be possibly protect the chondrocytes and maintain its differentiation.

The aim of the present work was attempted to reproduce experimentally OA in the knee joints of rats by means of intra-articular administration of MIA and to evaluate the impact of antioxidant on it and analyzing the process using the scanning electron microscope.

2. MATERIALS AND METHODS

2.1. Animals

The experiments were performed on healthy male Wistar rats of 10 weeks old and weighting 150-200 g. The rats were fed with standard laboratory diets, given water ad libitum and maintained under laboratory

conditions of temperature ($22\pm 3^{\circ}\text{C}$), with 12 h light and 12 h dark cycle. All experimental procedures involving the handling and treatment of animals were approved by the Ethical Committee of King Khalid University Medical School (Abha, KSA) and were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

2.2. Induction of OA

OA was induced chemically by injection of 2 mg of MIA (total volume of 50 μL) intra-articular through the patellar ligament on the right knee, using 26-gauge needle, while rat is under anesthesia (sodium thiopentone, 40 mg kg^{-1} , i.p.). The left knee joint (control) will be injected with saline (Bar-Yehuda *et al.*, 2009).

2.3. Experimental Design

After one week adaptations, the animal were classified and randomly allocated into 4 groups (n = 10) as follows:

2.4. Control Group (C)

Vitamin E group (E) treated group (600 mg Kg^{-1} body weight, i.m.), (Haidara *et al.*, 2006), 3 times per week for the whole period of the experiment.

Osteoarthritis group (OA): OA will be induced chemically by injection of MIA as stated above.

OA+Vitamin E (OAE) group: OA rats will be injected with both MIA and vitamin E, same doses as in previous groups.

At the end 8th weeks of the experiment, 5 mL retro-orbital blood samples will be obtained under anesthesia using 40 mg Kg^{-1} sodium thiopentone, i.p., after an overnight fast 3 mL will be collected into 3.8% Na citrate anticoagulant for plasma separation. While 2 mL will be collected into plain tubes, then allowed to clot for 20 min then centrifuged at 14000 rpm for 10 min for serum separation. Then plasma and serum will be stored at -80°C , for subsequent measurements of biochemical parameters. After withdrawal of the blood samples, the knee joints will be opened, dissected and will be fixed and kept for electro-microscopic examination. Samples will be assigned a second random number, thus to keep the study blinding.

2.5. Biochemical Parameters

We used rat ELISA kits for TNF- α (TSZ ELISA, Framingham, MA, USA), IL-6 (Ray Biotech Inc., Norcross, GA, USA). Serum SOD activity were measured using colorimetric assay kits (Cayman, Ann Arbor, MI, USA).

2.6. Scanning Electron Microscopy (SEM)

All of the articular acetabular cartilage bilaterally was fixed with 2.5% (wt/vol) sodium cacodylate-buffered glutaraldehyde, pH 7.2 at 4°C for 2 h. Samples were also post-fixed in 1% sodium cacodylate-buffered osmium tetroxide, pH 7.2 for 1 h. After washing and dehydration in ascending grades of ethanol, critical-point drying was performed using the EMITECH-K850 critical-point drying unit. The samples were mounted on aluminum stubs with double sided tape and silver glue and then sputter coated with gold by Boc Edwards Scan coat (AL-Shameri and Rong, 2009). The specimens were observed using a Jeol scanning electron microscope JSM-6390LV, Japan.

2.7. Statistical Analysis

Values are measured as mean \pm SD. Comparison of data was performed by using ANOVA test (analysis of variance test) using graph pad prism analysis software, version 5. Probability (P) values of <0.05 were considered to be significant.

3. RESULTS

3.1. Serum TNF- α

OA model rats exhibited significant increase in serum TNF- α levels (37.8 ± 2.9 ng/mL) as compared with control rats (18.4 ± 1.4) and vitamin E treated group (11.6 ± 1.7). Treating OA rats with vitamin E injection (600 mg kg^{-1}BW , 3 times/week) resulted in a significant reduction in serum TNF- α (27.4 ± 1.8) toward normal values when compared to OA model group. However, it is still higher than control and vitamin E treated groups **Fig. 1**.

3.2. Serum IL-6

OA model rats exhibited significant increase in serum IL-6 level (253 ± 34 pg/mL) as compared with control rats (121 ± 23) and vitamin E treated group (61 ± 13). Treating OA rats with vitamin E injections resulted in a significant reduction in serum IL-6 (186 ± 14) towards control values when compared to OA model group. However, it is still higher than control and vitamin E treated groups **Fig. 2**.

3.3 Serum SOD

OA model rats exhibited significant increase in serum SOD level (13.7 ± 0.6 U/mL) as compared with control rats (8.0 ± 0.6 U/mL) and vitamin E treated group (6.4 ± 0.7 U/mL).

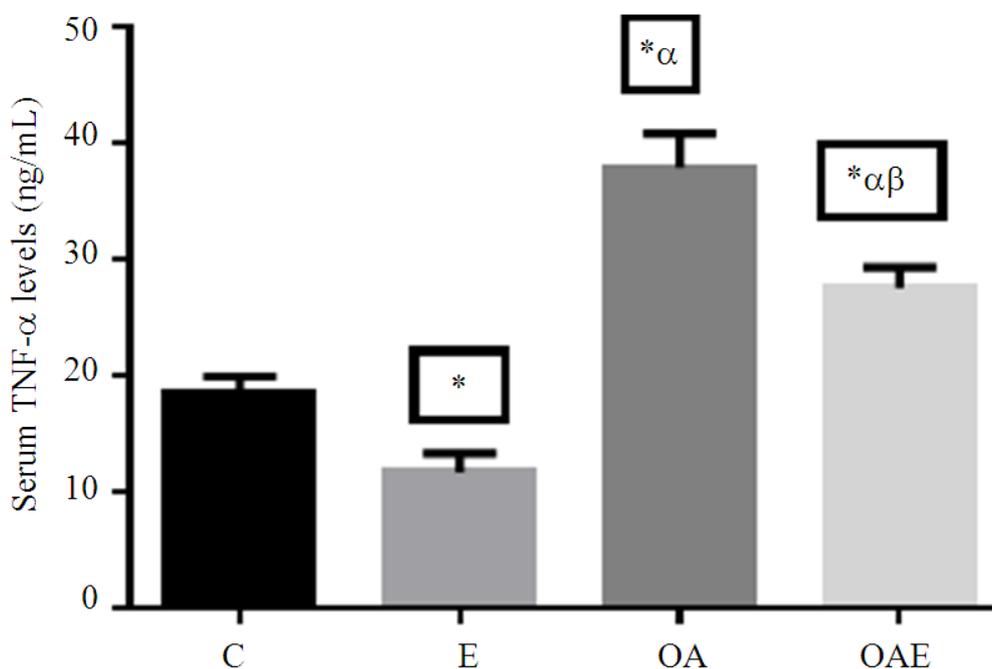


Fig. 1. Serum TNF- α in all groups. Control (C), vitamin E (E), Osteoarthritis (OA), Osteoarthritis and vitamin E (OAE). Values are expressed as mean \pm SD (n = 10). Values were considered significantly different at $p < 0.05$: * Significantly different compared to control group. α : Significantly different compared to vitamin E group. β : Significantly different compared to OA group

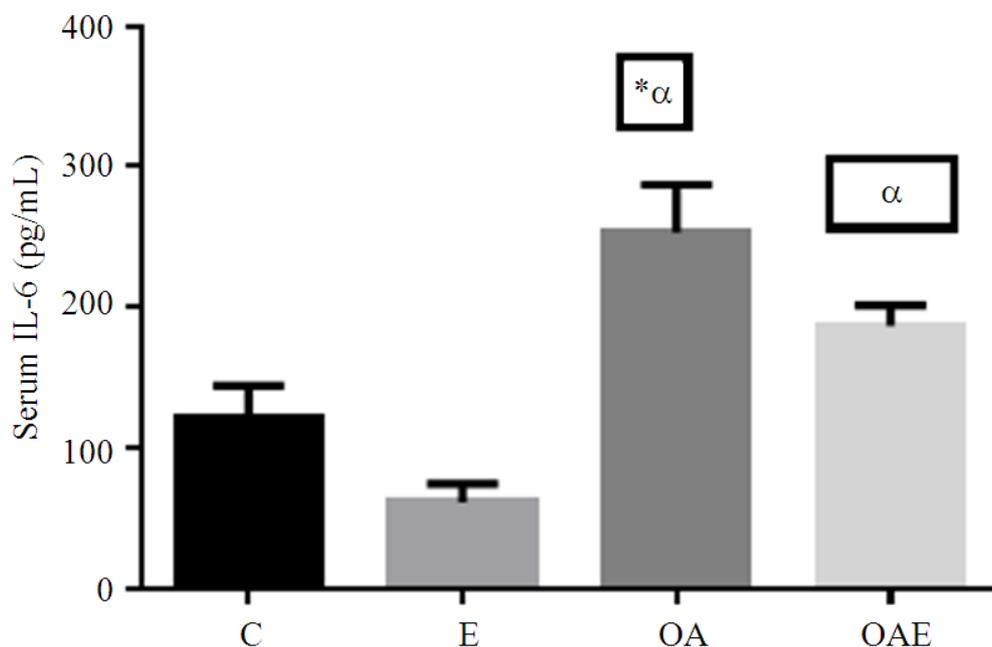


Fig. 2. Serum IL-6 in all groups. Control (C), vitamin E (E), Osteoarthritis (OA), Osteoarthritis and vitamin E (OAE). Values are expressed as mean \pm SD (n = 10). Values were considered significantly different at $p < 0.05$: * Significantly different compared to control group. α : Significantly different compared to vitamin E group

Treating OA rats with vitamin E injections resulted in a significant reduction in serum SOD (10 ± 0.4) towards normal values when compared to OA model group. However, it is still higher than control and vitamin E treated groups **Fig. 3**.

3.4. Scanning Electron Microscopy (SEM) Results of Knee Joint

3.4.1. Electron Micrograph of Cartilage Cells and Territorial Matrix

Chondrocytes of knee joints of control **Fig. 4** and vitamin E treated **Fig. 5** groups in the articular

cartilage appears normal with leaving Lacunae (LC) between normal Chondrocytes (C) and the chondrocytes are surrounded by normal Territorial Matrix (TM). **Fig. 6** showed SEM of osteoarthritis-induced rat articular cartilage where there is damaged leaving Lacunae (LC) and Chondrocytes (C). The chondrocytes surrounded by destroyed Territorial Matrix (TM). A scanning electron micrograph of osteoarthritis rat articular cartilage treated by vitamin-E showed focal damage of leaving Lacunae (LC) and Chondrocytes (C) which surrounded by less damaged and improved Territorial Matrix (TM) **Fig. 7**.

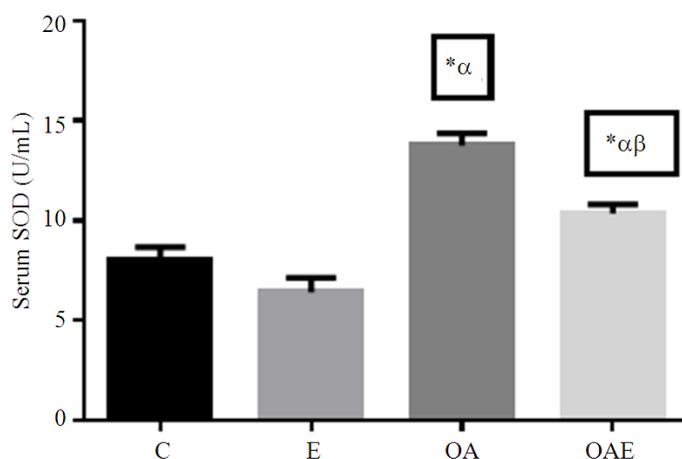


Fig. 3. Serum SOD in all groups. Control (C), vitamin E (E), Osteoarthritis (OA), Osteoarthritis and vitamin E (OAE). Values are expressed as mean \pm SD (n = 10). Values were considered significantly different at $p < 0.05$: * Significantly different compared to control group. α: Significantly different compared to vitamin E group. β: Significantly different compared to OA group

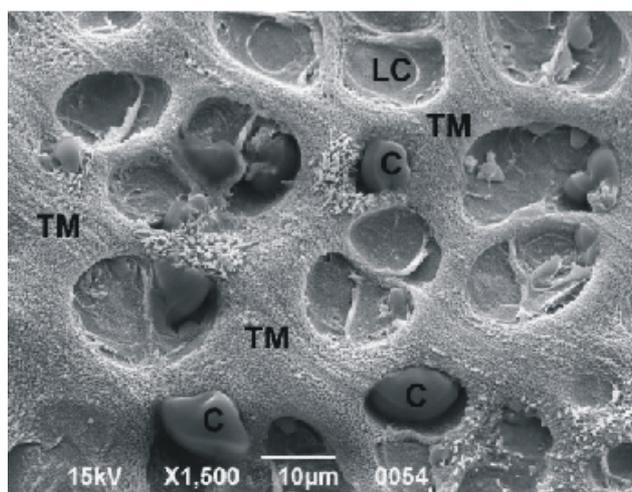


Fig. 4. Electron micrograph of control rat articular cartilage showing leaving Lacunae (LC) between Chondrocytes (C). The chondrocytes surrounded by Territorial Matrix (TM)

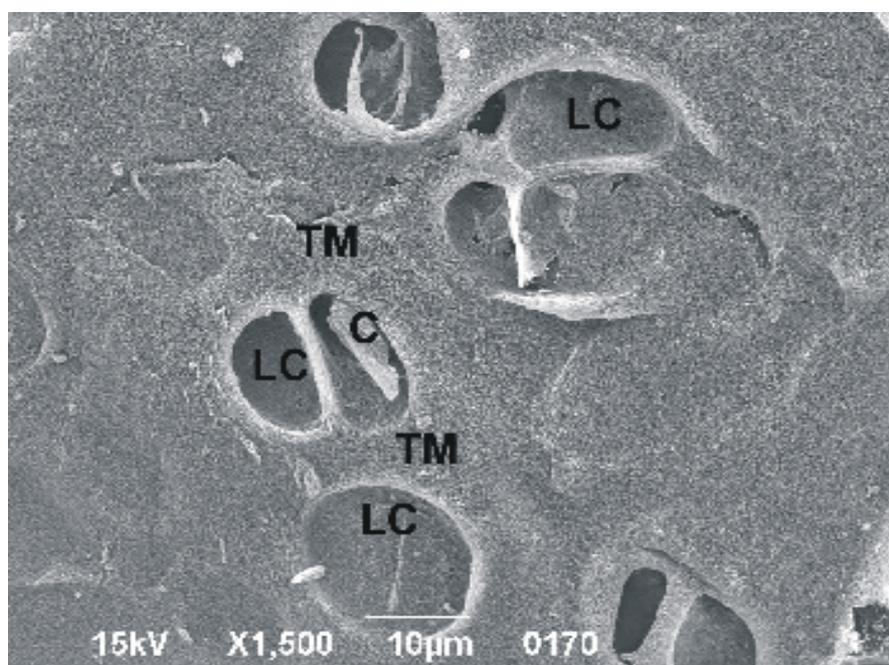


Fig. 5. Electron micrograph of micrograph of vitamin-E treated rat articular cartilage showing leaving Lacunae (LC) between Chondrocytes (C). The chondrocytes surrounded by normal Territorial Matrix (TM)

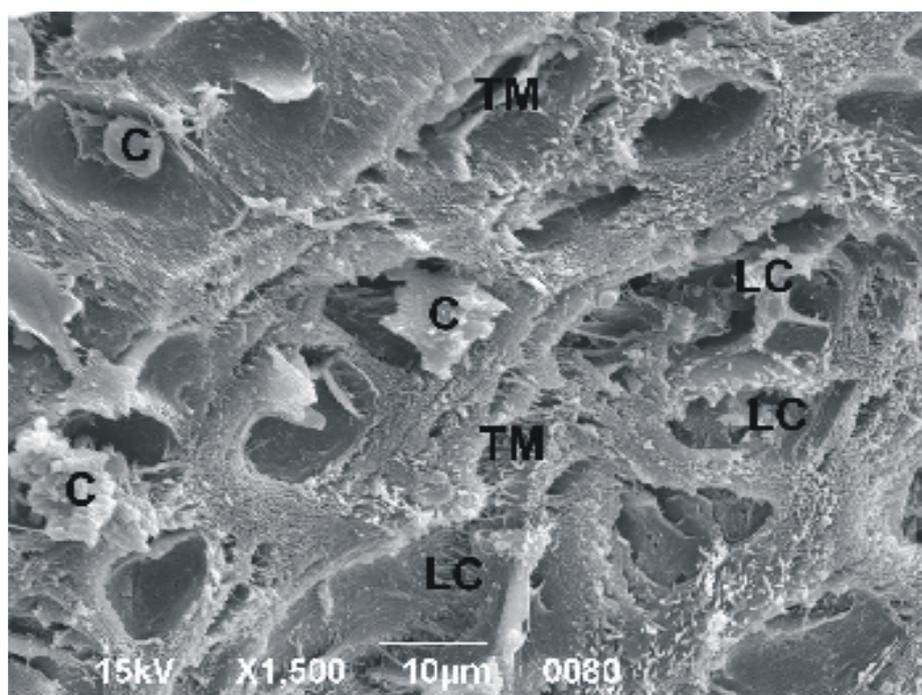


Fig. 6. Electron micrograph of osteoarthritis rat articular cartilage showing massive damaged leaving Lacunae (LC) and Chondrocytes (C). The chondrocytes surrounded by destroyed Territorial Matrix (TM)

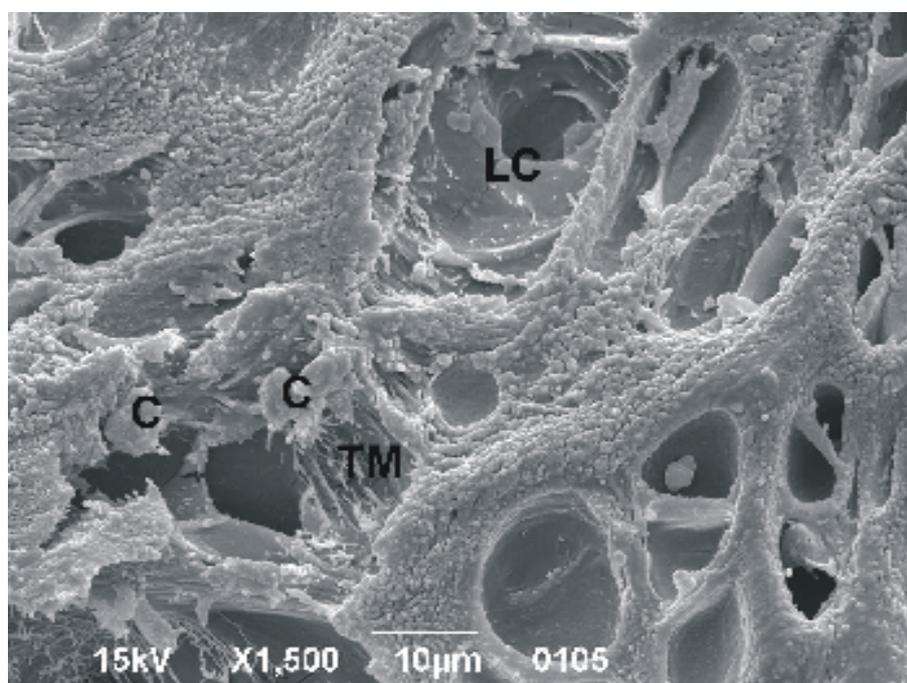


Fig. 7. Electron micrograph of osteoarthritis rat articular cartilage treated by vitamin-E showing focal damage of leaving Lacunae (LC) and Chondrocytes (C) which surrounded by less damaged and improved Territorial Matrix (TM)

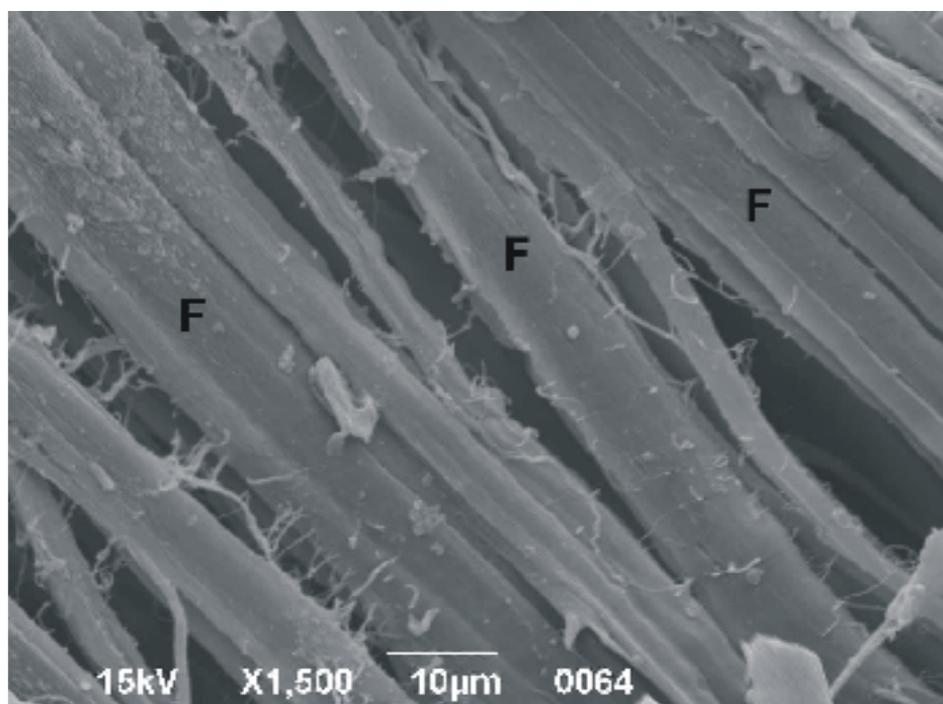


Fig. 8. Electron micrograph of control rat collagen fiber showing normal Fibrils (F) form sheets

3.5. Electron Micrograph of Collagen Fibers of Knee Joint

Figure 8 and 9 showed collagen fibers of control and vitamin E treated rats, collagen Fibrils (F) are normal forming regular sheets. Figure 10 represents SEM of

osteoarthritis induced rats, which showed massive damage of collagen Fibrils sheets (F). Figure 11 represents SEM of osteoarthritis rat collagen fiber treated by vitamin E where it showed improvement and slight Focal deformation (F) and intact (arrows) fibrils sheets.

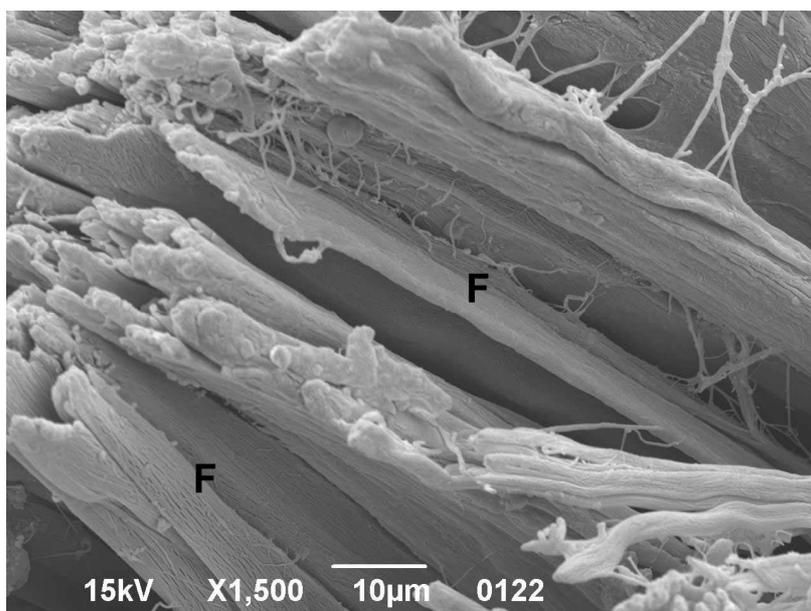


Fig. 9. Electron micrograph of Vitamin E treated rat collagen fiber showing normal Fibrils (F) form sheets

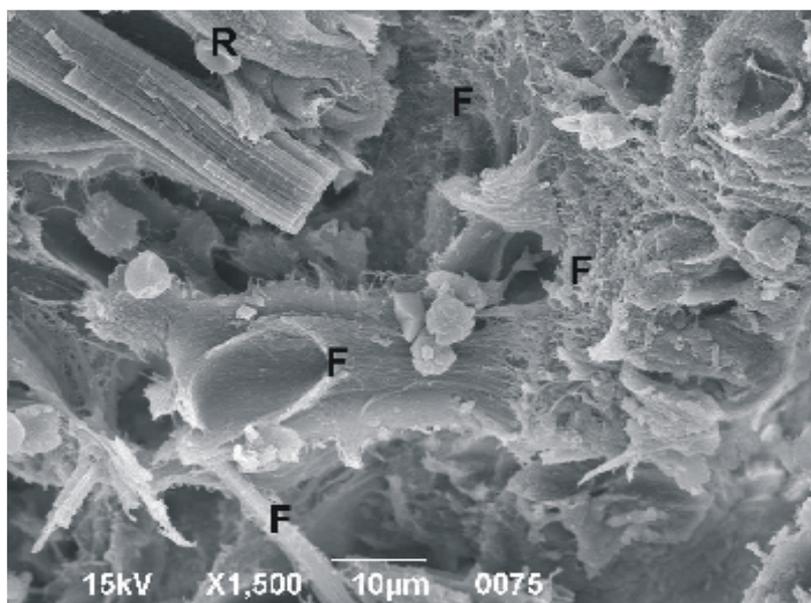


Fig. 10. Electron micrograph of osteoarthritis rat collagen fiber showing massive damage of fibrils sheets (F)

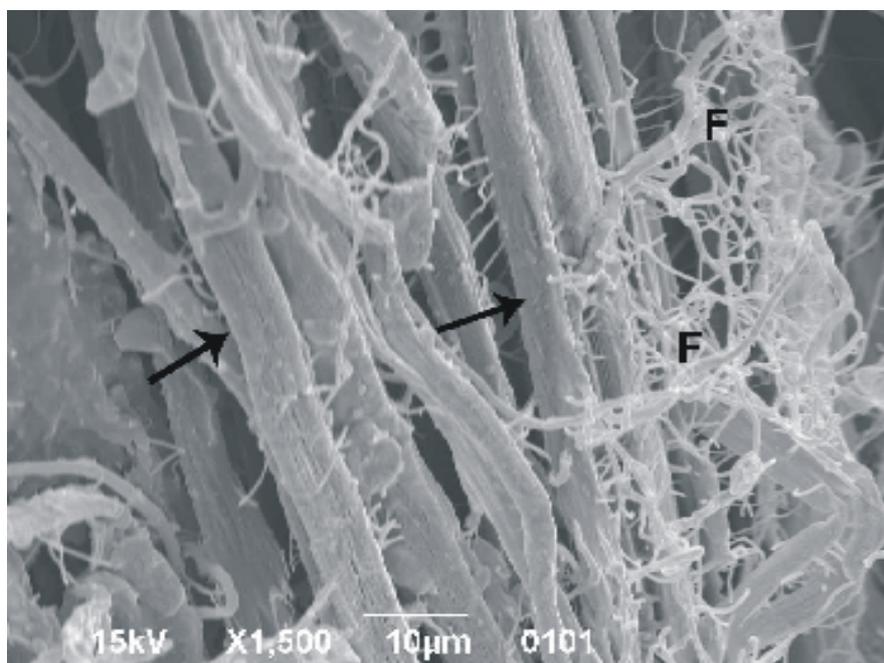


Fig. 11. Electron micrograph of osteoarthritis rat collagen fiber treated by vitamin E showing improvement and slight focal deformation (F) and intact (arrows) fibrils sheets

4. DISCUSSION

In the current study, we used a well-established animal model of OA, which was induced by the intraarticular injection of MIA into rat knee joints. MIA disrupts cartilage metabolism, which leads to chondrocyte death and subchondral bone lesions that are consistent with the pathologic changes seen in OA in humans (Moon *et al.*, 2014). Treatment with vitamin E counteracted this effect which is apparent with SEM examination.

The molecular mechanism of action entailed decreased inflammatory markers and proven antioxidant effect through increased SOD activity. The antioxidant effect of vitamin E has previously been proved in different experimental animal models, both in mice and in rats (Haidara *et al.*, 2006). These data prompted that administration of vitamin E three times per week resulted in a steady-state blood level of the vitamin and proven effective as antioxidant (Haidara *et al.*, 2010). Vitamin E administration improved the structure of the affected joints, thereby alleviating the development of the disease. More specifically, the impact of vitamin E on the development of OA was manifested by decrease of inflammatory markers (TNF- α and IL-6). In support of this improvement

were the SEM findings which showed that vitamin E treatment preserved the cartilage and associated with focal damage rather than massive damage of leaving Lacunae (LC) and Chondrocytes (C) encountered in OA induced rats. These chondrocytes are also surrounded by less damaged and improved Territorial Matrix (TM). Thus EMS documented the beneficial effects of vitamin E on different components of the joint.

It is generally accepted that pro-inflammatory cytokines, especially TNF- α , are critical for the pathophysiology and subsequent complications of exaggerated inflammatory response. TNF- α alone or in combination with IL-6 and complement is responsible for neutrophil transmigration and sequestration in tissues during endotoxemia and sepsis. Neutrophils attack tissues and cause severe cell necrosis TNF- α also leads to the production of the nuclear factor NF- κ B in endothelial cells during inflammation (Huang *et al.*, 2014). NF- κ B is then responsible for the transcriptional activation of a number of pro-inflammatory genes such as nitric oxide synthase and adhesion molecules such as Intercellular adhesion molecule-1, selectins and Vascular cell adhesion molecule-1 (Huang *et al.*, 2014).

The mechanisms involved in the effects of vitamin E include the deregulation of NF- κ B signaling pathway,

which is known to play a major role in the biomechanical pathways involved with the pathogenesis of OA. It has previously been shown that the NF- κ B signaling pathway mediates the progression of extracellular matrix damage and cartilage destruction. NF- κ B was also shown to induce the expression of pro-inflammatory cytokines, chemokines and matrix metalloproteinase by human articular chondrocytes (Pulai *et al.*, 2005). The deregulation of the NF- κ B signaling pathway has previously been demonstrated in animal models of MIA induced arthritis (Arii *et al.*, 2008).

Indeed, our study showed that vitamin E administration is accompanied by a decrease in the levels of the inflammatory markers (IL-6 and TNF- α) leading to an anti-inflammatory effect and improvement in ultra-structure components of the knee joint. We have demonstrated in the present study that treatment of OA with vitamin E, an oxidative stress antagonist, is effective. The mechanism involves anti-inflammatory and antioxidant effects. Based on the data presented vitamin E appears to alter the course of the disease and may be suggested to be an adjuvant OA drug.

5. CONCLUSION

In conclusion, we have documented that administration of vitamin E in the doses and durations suggested resulted in a significant reduction of the inflammatory markers and oxidative stress parameters and improved ultra-structure of the knee joint in acute OA animal model. However, their value in predicting the effectiveness of treatment strategies in clinical trials has remained controversial. In fact, clinical trials are essential because animal studies do not predict with sufficient certainty what will happen in humans. Clinical trial is required to prove the efficacy of administration of vitamin E alone or as an adjuvant treatment during inflammatory conditions of joints. In view of the emerging role of antioxidants during inflammation, it may be worthwhile to study also the effects of treatment on pro-inflammatory cytokine activity.

Limitation of the current study: Measurements and intervention were made without blinding of the researcher to the experimental group, which has the potential for bias. However, potential bias was minimized by random assignment of participants and monitoring of consistent protocol by the principal investigator. A short post-operative follow-up period of eight weeks is another limitation for a number of reasons. At this post-operative time point, OA changes is still occurring, longer follow up at 3 and 6 months following surgery would uncover more promising results.

6. DECLARATION OF INTEREST

The researchers report no conflict of interest.

7. ACKNOWLEDGMENT

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