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Comparative effects of intra-articular administration of vitamin C, vitamin D, and hyaluronic acid in an experimental osteoarthritis model in rats

[*Comparação dos efeitos das aplicações intra-articulares de vitamina C, vitamina D e ácido hialurônico em um modelo experimental de osteoartrite em ratos*]

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ABSTRACT

Osteoarthritis (OA) is a chronic disease characterized by inflammation or cartilage deterioration in joints, leading to debilitating, movement-associated pain and degenerative changes in joint cartilage, bone, and other surrounding tissues, caused by factors such as joint injury, aging, obesity, and heredity. Various agents are used in OA treatment. In this study, hyaluronic acid, vitamin C and vitamin D were used in different combinations. A total of 32 rats were used in the study. After osteoarthritis was experimentally induced in the rats under general anesthesia, they were randomly divided into eight groups. Then, the preparations were administered intra-articularly as a single dose according to the groups. The study was terminated after 21 days. The tissues collected post-study were examined histopathologically. According to the histopathological findings of the study, the preparations provided mild cartilage tissue regeneration when applied individually, moderate regeneration when used in pairs, and significant regeneration and new bone trabecula formation when used in a triple combination.

Keywords: hyaluronic acid, osteoarthritis, vitamin C, vitamin D

RESUMO

A osteoartrite (OA) é uma doença crônica caracterizada pela inflamação ou deterioração da cartilagem nas articulações, causando dor debilitante associada ao movimento e alterações degenerativas na cartilagem articular, no osso e em outros tecidos circundantes, causadas por fatores como lesão articular, envelhecimento, obesidade e hereditariedade. Vários agentes são usados no tratamento de OA. Neste estudo, ácido hialurônico, vitamina C e vitamina D foram usados em diferentes combinações. Um total de 32 ratos foram usados no estudo. Após a osteoartrite ter sido induzida experimentalmente nos ratos sob anestesia geral, eles foram divididos aleatoriamente em oito grupos. Em seguida, as preparações foram administradas por via intra-articular em dose única de acordo com os grupos. O estudo foi encerrado após 21 dias. Os tecidos coletados pós-estudo foram examinados histopatologicamente. De acordo com os achados histopatológicos do estudo, as preparações proporcionaram regeneração leve do tecido cartilaginoso quando aplicadas individualmente, regeneração moderada quando usadas em pares e regeneração significativa e formação de novas trabéculas ósseas quando usadas em uma combinação tripla.

Palavras-chave: ácido hialurônico, osteoartrite, vitamina C, vitamina D

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INTRODUCTION

Osteoarthritis (OA) is a chronic disease characterized by inflammation or degeneration of joint cartilage, leading to debilitating pain associated with movement. Degenerative changes in bone, joint cartilage, and other surrounding tissues, including ligament calcification, subchondral bone thickening, osteophyte development, synovial inflammation, and gradual cartilage loss, are some of its symptoms (Pei *et al*., 2019; He *et al*., 2020b; Yabas *et al*., 2021).

OA has a multifactorial etiology, with contributing factors including joint injury, aging, obesity, and heredity. Although the molecular mechanisms underlying OA pathogenesis remain incompletely understood, both chondrocytes and inflammation are thought to play critical roles. Analgesics and non-steroidal anti-inflammatory medications are currently available therapies for OA; nevertheless, they are symptomatic rather than curative, with the only goal of managing symptoms and reducing pain. Total knee or hip replacements are examples of surgical procedures that can reduce pain and deformity, but they can also result in postoperative problems (He *et al*., 2020a). In addition to all this, there are OA treatments that aim not only to treat joint pain and inflammation, but also to increase the anabolic activity of chondrocytes and improve tissue repair by reducing tissue degeneration (Parlak *et al*., 2022). In experimental OA models, a variety of agents have been employed in OA treatment, including gold nanoparticles combined with hyaluronic acid, magnesium chloride, mesenchymal stem cells, hydrogen sulfide, and platelet-rich plasma (Yao *et al*., 2019; Jayaram *et al*., 2020; Santos Filho *et al*., 2021; Lin *et al*., 2022; Lendoiro-Cino *et al*., 2023).

The primary natural property of Vitamin C is its ability to act as a potent antioxidant, reducing and stabilizing potentially harmful free radical compounds. Free radicals (including superoxide, hydrogen peroxide, and peroxynitrite) are known to generate and accumulate within chondrocytes, which is one of the primary causes of age-related cartilage degeneration OA. More precisely, these reactive oxygen species cause human chondrocytes to become genomically unstable, which reduces the amount of viable cartilage. Given this knowledge, antioxidants such as vitamin C may be protective against chondrocyte dysfunction brought on by oxidative stress (Dunlap *et al*., 2021).

Vitamin D is essential for the metabolism of cartilage. It is widely recognized for its vital roles in the metabolism of calcium and phosphorus as well as its importance in several bone and mineral diseases, including rickets and osteoporosis. By attaching itself to vitamin D receptors, vitamin D also controls immunological response, cell division, and proliferation. By affecting the synthesis of collagen and proteoglycans as well as plasminogen activator activity, vitamin D is believed to regulate chondrocyte expression in various regions of growth plate cartilage (Busa *et al*., 2023).

Hyaluronic acid (HA), sometimes referred to as hyaluronan or hyaluronate, is a naturally occurring, non-sulfated glycosaminoglycan that is present in a variety of tissues and fluids but is most commonly found in synovial fluid and joint cartilage. Synoviocytes, fibroblasts, and chondrocytes produce HA, which has a variety of physicochemical characteristics. The amount of HA in various joints and species varies greatly. HA contributes to the viscoelasticity and lubrication of normal joint fluid, which are important aspects of its biomechanics. The concentration and molecular weight (MW) of HA both decline with aging-related OA. As a result, HA has long been utilized to treat OA in people, horses, and dogs. HA uses a variety of receptors, enzymes, and other metabolic pathways to provide its anti-arthritic actions. Additionally, HA is employed in the treatment of ophthalmic, dermal, burn, wound repair, and other health conditions (Gupta *et al*., 2019).

The aim of this study is to compare the efficacy of intra-articular injections of vitamin C, vitamin D, and hyaluronic acid in an experimental osteoarthritis model in rats.

MATERIALS AND METHODS

The study was conducted in accordance with the approval of the Animal Experiments Local Ethics Committee of Sivas Cumhuriyet University,

under the letter dated 12.05.2023 with the number 65202830-050.04.04-733.

A total of 32 female rats were used for the study. From these rats, 4 animals were randomly selected for each group (Metineren and Dülgeroğlu, 2019); the groups included: Control, Vitamin C, Vitamin D, Hyaluronic Acid, Vitamin C + Vitamin D, Hyaluronic Acid + Vitamin C, Hyaluronic Acid + Vitamin D, and Hyaluronic Acid + Vitamin C + Vitamin D.

For anesthesia, Xylazine hydrochloride (Rhompun, Bayer, Istanbul) at a dose of 3 mg/kg and Ketamine hydrochloride (Ketalar, Eczacıbaşı, Istanbul) at 90 mg/kg were administered. Vitamin C (TURKTIPSAN VİTAMİN C®, 500mg/5ml TURKTIPSAN) at 4mg/kg (Azizi *et al*., 2019), Vitamin D (DEVIT-3®, 200.000 IU/10 ml, Deva) at 1000 IU/rat (The recommended dosage range of DEVIT-3 for adults over 18 years of age is 600-1500 I.U./day.), and Hyaluronic acid (Ostenil Plus®, 40mg/2ml, Bio-Gen) at 4 mg/kg (Kim *et al*., 2019) were administered intra-articularly.

After the rats were induced with general anesthesia, the surgical site was shaved and disinfected. A longitudinal incision was made laterally to the patella to expose the femoral condyles. The patella was laterally displaced to reveal the intercondylar notch, and an osteochondral defect was created using a 1.50 mm diameter Kirschner wire to a depth of 2 mm (After determining 2 mm on the Kirschner wire with a caliper, the wire was marked, and the depth measurement was provided.). This procedure was performed for the right knee of each animal (Metineren and Dülgeroğlu, 2019) (Fig. 1).

A total of 32 rats were used in the study, and they were randomly divided into 8 groups, each containing 4 rats. The groups were named as follows:

1. Control group: In this group, osteochondral defects were created as described. No further treatment was administered.

2. Vitamin C group: In this group, osteochondral defects were created as described. Afterward, a single dose of intra-articular Vitamin C was administered.

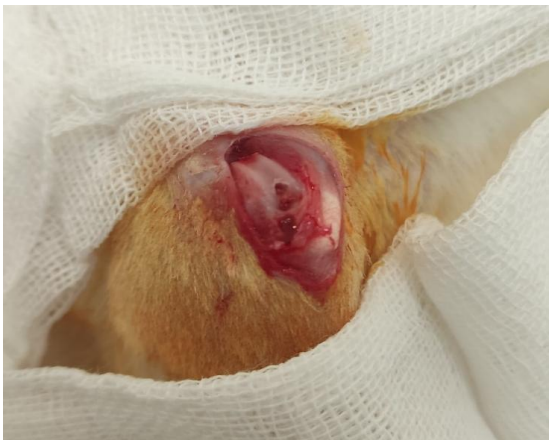

Figure 1. A Kirschner wire was used to induce osteochondral defects at the right knee defects and femoral condyles.

3. Vitamin D group: In this group, osteochondral defects were created as described. Afterward, a single dose of intra-articular Vitamin D was administered.

4. HA group: In this group, osteochondral defects were created as described. Afterward, a single dose of intra-articular Hyaluronic Acid (HA) was administered.

5. Vitamin C + Vitamin D group: In this group, osteochondral defects were created as described. Afterward, a single dose of intra-articular C + D Vitamin was administered.

6. HA + Vitamin C group: In this group, osteochondral defects were created as described. Afterward, a single dose of intra-articular Hyaluronic Acid + Vitamin C was administered.

7. HA + Vitamin D group: In this group, osteochondral defects were created as described. Afterward, a single dose of intra-articular Hyaluronic Acid + Vitamin D was administered.

8. HA + Vitamin C + Vitamin D group: In this group, osteochondral defects were created as described. Afterward, a single dose of intra-articular Hyaluronic Acid + Vitamin C + Vitamin D was administered.

The study was concluded after 21 days. The rats were euthanized through exsanguination (bleeding out) under general anesthesia following surgery.

Bone tissue samples taken for histopathological examination were fixed in 10% formalin solution for 48 hours. After fixation, the samples were decalcified in Osteosot (Merc, HC313331, Germany) until softened. Once the tissues were softened, they were washed in running tap water for 24 hours. After completing the routine bone tissue processing, the samples were embedded in paraffin blocks. Sections of 4 microns in thickness were obtained from each block, stained with hematoxylin and eosin, and examined under a light microscope.

RESULTS

In the histopathological examination of the bone tissue in the defect area, the following observations were made:
Control group: Mild angiogenesis was observed, the defect was organized with fibrous tissue, cartilage regeneration had just begun, and very slight bone trabecular formation had started (Figure 2-A).

Vitamin C group: A dense fibrous tissue formation in the defect area, moderate angiogenesis, moderate inflammation, and the onset of cartilage regeneration were identified (Figure 2-B).

Vitamin D group: Dense fibrous tissue formation in the defect area, severe angiogenesis, severe inflammation, and the beginning of cartilage regeneration were observed (Figure 2-C).

HA group: Dense fibrous tissue formation in the defect area, mild angiogenesis, and the onset of cartilage regeneration were observed (Figure 2-D).

Vitamin C + Vitamin D group: Dense connective tissue formation in the defect area, conversion of fibrous tissue into chondrocytes, severe angiogenesis, and moderate cartilage regeneration were identified (Figure 2-E).

HA + Vitamin C group: Conversion of dense connective tissue cells into chondrocytes in the defect area, intense angiogenesis, moderate cartilage regeneration, and new bone trabecular formation were observed (Figure 2-F).

HA + Vitamin D group: Dense connective tissue formation in the defect area, new bone trabecular formation, conversion of fibrous tissue into chondrocytes, and intense angiogenesis were noted (Figure 2-G).

HA + Vitamin C + Vitamin D group: The defect area had become shallower, cartilage regeneration was intense, and fibrous tissue had started to transform into chondro-osteocytes (Figure 2-H). A statistically significant difference ($p<0.05$) was observed when compared to the control group.

Table 1. Scoring of histopathological findings observed in the defect area

Group	Cartilage regeneration	Bone trabeculae formation	Angiogenesis	Inflammation
Control	+	+	++	+++
VitC	++	+	++	++
VitD	+	+	+++	+++
HA	+	+	++	+++
VitC + VitD	+++	++	+++	++
HA + VitC	++	++	+++	++
HA + VitD	++	++	+++	++
HA + VitC + VitD	+++	+++	++	+

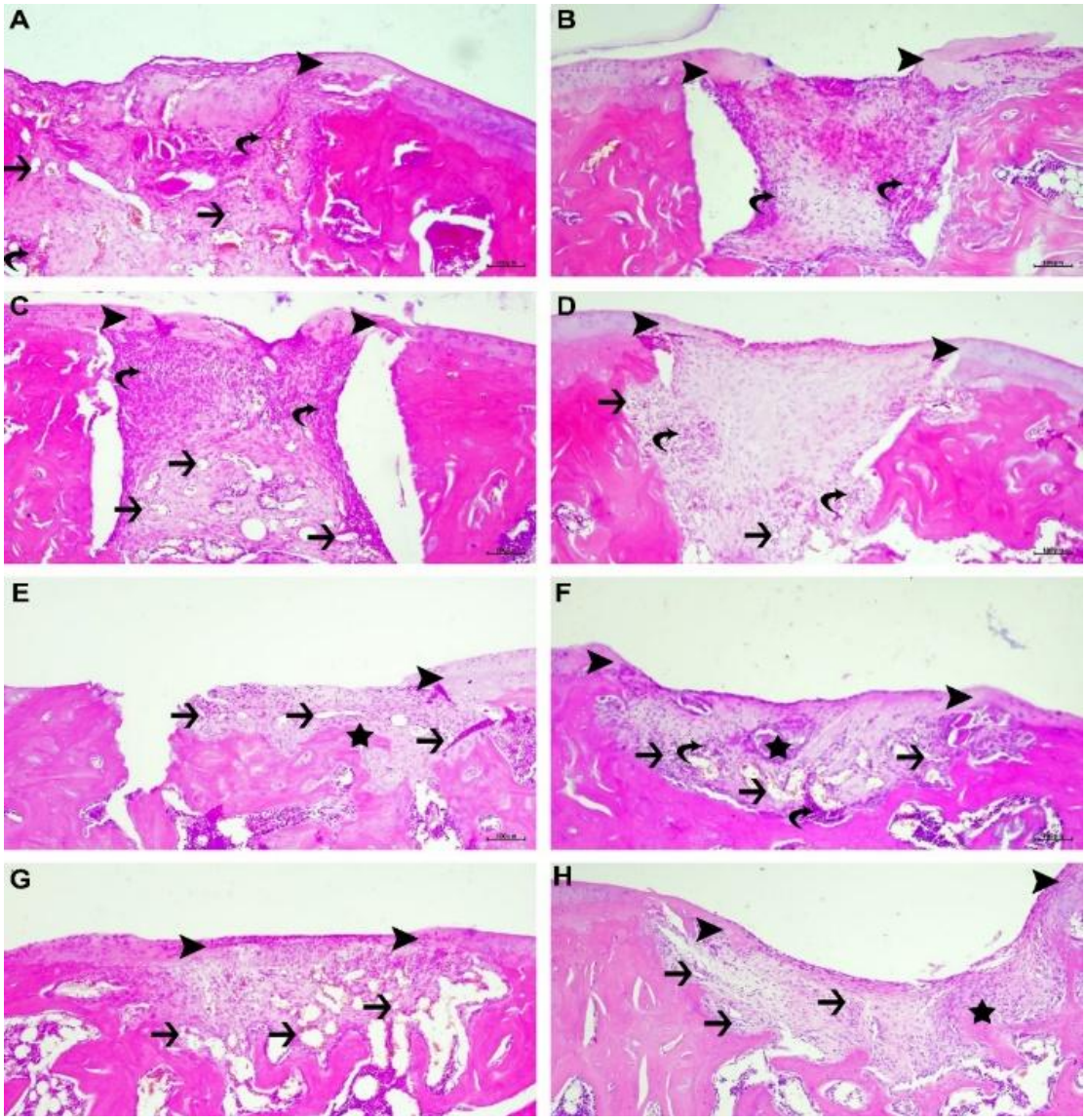

Figure 2: Bone tissue, cartilage regeneration (arrowheads), angiogenesis (arrows), inflammation (curved arrows), new bone trabecular formation (stars). Control group (A), Vitamin C group (B), Vitamin D group (C), HA group (D), Vitamin C + Vitamin D group (E), HA + Vitamin C group (F), HA + Vitamin D group (G), HA + Vitamin C + Vitamin D group (H), H&E, Bar: 40 µm.

DISCUSSION

Degeneration of articular cartilage, thickening of subchondral bone, formation of osteophytes, varying degrees of synovial inflammation, ligament degeneration, hypertrophy of the joint capsule, and alterations in periarticular muscles, nerves, bursae, and local fat pads are all characteristics of OA, a joint organ insufficiency that affects all tissues inside and around the joint. Cell death, matrix calcification, vascular invasion, fibrillation and erosion of the joint surface, and the breakdown of the extracellular matrix (ECM), which is rich in collagen and proteoglycans, are all consequences of the irregularity brought on by the presence of different biofactors in OA (He *et al*., 2020b).

A powerful antioxidant, vitamin C also plays a crucial role in the post-translational modification of collagen synthesis and the stability of the collagen network (Yao *et al*., 2021). Adequate levels of vitamin C can support collagen synthesis in tendons and muscles, as well as bone

density, potentially reducing the degree of structural changes associated with existing osteoarthritis pathology. Vitamin C also has the capacity to reduce the risk of joint injury, pain, inflammation, and muscle atrophy, and to enhance tendon growth, healing processes, and bone health (Marks, 2018). In a study by Azizi *et al*. (2019), after the formation of osteoarthritis in rats, the application of vitamin C and serum rich in growth factors (SRGF) was observed to enhance cartilage regeneration. The results of this study are similar to those of our own research.

Skeletal health and the proper metabolism of bone and cartilage depend on vitamin D. In addition to oxidative stress damage and mitochondrial abnormalities that worsen OA illness and increase pain, low vitamin D levels have also been linked to disruptions in cartilage structure and function, muscle and bone properties, bone mass, osteoblastic activity, calcium and phosphorus metabolism, and joint cartilage cycles. Oxidative stress conditions and changes in mitochondrial membrane potential affect vitamin D status and the severity of OA. Vitamin D receptors play a crucial role in regulating vitamin D concentration in chondrocytes to maintain a stable redox environment. Furthermore, it has been shown that the amount of vitamin D varies according to gender and age, accompanying the chronic inflammation and pain index in joints (Marks, 2021; Amirkhizi *et al*., 2022; Busa *et al*., 2023). In a study by Neve *et al*. (2013), an increase in angiogenesis was observed after 12 days of vitamin D supplementation in patients with osteoarthritis. Additionally, Busa *et al*. (2023) applied vitamin D in an experimental osteoarthritis model in rats, and it was found that cartilage damage was reduced following the application. These results are in line with the findings of the present study.

Because of its non-immunogenic qualities, regulated biodegradability, friendly polymerization chemistry, and numerous reactive sites, HA is a useful ingredient for making hydrogels that promote healing (Bowman *et al*., 2018). Because the synovial fluid in osteoarthritic joints has lower quantities of HA than in healthy joints, its viscoelastic qualities can be restored by intra-articular therapy with exogenous HA. Exogenous HA has been shown to have a variety of physiological effects in both in vitro and in vivo studies, which may mitigate the mechanisms underlying the pathophysiology of OA. Exogenous HA can, in fact, promote regeneration, stop cartilage deterioration, and increase chondrocyte production of endogenous HA and proteoglycans. Furthermore, it can reduce the production of pro-inflammatory mediators and matrix metalloproteinases, as well as decrease nerve stimulation and sensitivity associated with OA pain (Migliore and Procopio, 2015). In a study by Tsai *et al*. (2012), hyaluronic acid was shown to protect against cartilage degeneration in an osteoarthritis model in rats. In the study by Abd Alhalim *et al*. (2021), osteoarthritis was experimentally induced in rats. After the formation of osteoarthritis, hyaluronic acid was applied alone and in combination with bone marrow-derived mesenchymal stem cells (BM-MSCs). The results showed that hyaluronic acid alone provided moderate protection against osteoarthritis, while its combination with BM-MSCs offered better protection. These findings are consistent with the results of our study, where hyaluronic acid alone provided moderate protection, but its combination with C and D vitamins led to much better protection against osteoarthritis. These data align with those of other studies.

CONCLUSION

In this experimental OA study, vitamin C, vitamin D, and HA were used intra-articularly, both alone and in combination. According to the histopathological findings of the study, when the treatments were applied alone, cartilage regeneration and the formation of new bone trabeculae had just begun. When the treatments were used in dual combinations, moderate cartilage regeneration and bone trabeculae formation were observed. In the case of triple combinations, severe cartilage regeneration and significant increases in bone trabeculae formation were identified. Although intra-articular HA treatments provided protective effects against osteoarthritis and prevented its progression, they were not sufficient on their own. Therefore, combining HA treatments with other preparations that could contribute to cartilage regeneration, bone trabecula formation, and reduction of inflammation would be more effective in managing the process in individuals

with osteoarthritis. Further studies are needed to develop new strategies.

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TS designed the study. TS and HOG performed the research, TS, VÖ, and HOG analysed the data, TS, FY, HOG, VÖ, HHA and İA wrote the paper.

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